UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

Understanding how capping of actin filaments prevents epithelium to mesenchymal like transitions in genetically defined epithelial tissues

Sofia Raquel Paulo Rebelo

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO
2007

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

Understanding how capping of actin filaments prevents epithelium to mesenchymal like transitions in genetically defined epithelial tissues

Sofia Raquel Paulo Rebelo

Dissertação de mestrado orientada por: Doutora Florence Janody[1]
Orientadora interna: Prof. Doutora Gabriela Rodrigues[2]

[1] Instituto Gulbenkian de Ciência, Oeiras.
[2] Faculdade de Ciências da Universidade de Lisboa, Lisboa.

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO
2007

ACKNOWLEDGEMENTS

The past year has been crucial for my formation as an individual and as a beginner in the scientific world. There are several people that have been really important during this period, to whom I would like to thank:

First, I would like to give a special thanks to my supervisor, Florence Janody. Not only for being available and supportive during the entire period of training but also for creating a respectful and friendly environment in lab, which has been crucial to the development of my work. I must also thank her for giving me such a beautiful project to work in during my master thesis and for the permanent motivation.

To my internal supervisor, Dr. Gabriela Rodrigues, for being as helpful as she could be whenever I needed.

To Beatriz.....I have no words to say how thankful I am. For the support and permanent motivation, for teaching me how to work and for being the wonderful person she is. Thanks a lot!

To Gaspar, the most promising scientist I know! Thanks for everything, especially for the enlightening discussions we have and for the comments on my thesis.

To Maurícia Cristina, for making my lab work more fun and easy (and for being a great matrecos-partner).

To Emília Pomba, not only for the scientific help on molecular biology, but also for being a very good friend (and for being my thesis-writing partner).

To the *Drosophila* community at the IGC, who have taught me a lot and make the fly work a very nice moment.

To Dr. Gabriel Martins, for helping me with the confocal imaging.

To all the people that I am not mentioning but that have contributed in any way to my work and to my life.

And to my family, for being always there.

Thanks to all of you!

LIST OF ABREVIATIONS

ABP	Actin Binding Proteins
AJ	Adherens Junctions
AP	Anterior/posterior
Arm	Armadillo (ß-catenin)
bp	base pair
CP	Capping protein
Cpa	Capping protein a
Cpb	Capping protein ß
Crb	Crumbs
D/V	Dorso-ventral
Dlg	Discs large
ECM	Extracelular matrix
EMT	Epithelium to mesenchymal transition
FA	Focal adhesions
FACS	Fluorescence activated cell sorting
F-actin	Filamentous actin
FAK	Focal adhesion kinase
IR	Inverted repeat
JNK	Jun N-terminal Kinase
Lgl	Lethal giant larvae
PCR	Polimerase chain reaction
RNAi	RNA interference
RT	Room temperature
SAR	Sub apical region
Scrib	Scribble
SJ	Septate junctions
TSG	Tumor supressor gene
Vg	Vestigial

ABSTRACT

The actin cytoskeleton has a central role in controlling cell shape and mobility. In epithelia, a circumferential band of actin filaments provides the structural support for cell-cell junctions. When the strength of the epithelium is compromised, cells may undergo epithelium to mesenchymal transition, escape size-control mechanisms, evade cell death and finally acquire the ability to migrate. These features recapitulate all of the hallmarks that characterize cancer malignancy. Interestingly, clones of cells mutant for either subunits of the *capping protein ab* heterodimer (*CP*), induced in a heterozygous wild-type background, are extruded from the wing blade epithelium and die. However, depleting the β subunit (*cpb*) by RNA interference (RNAi) in the whole blade leads to a different outcome: epithelium polarity is strongly affected, few apoptotic cells can be observed, while many seem to overproliferate. This differential behavior is unlikely to be due to a dosage effect since RNAi induced-*cpb* depletion in restricted wing blade domains also induces cell extrusion and death. This suggests that CP prevents tumorigenesis of wing blade cells. However, when *CP* mutant cells are adjacent to wild-type wing blade neighboring cells, the latest eliminate mutant cells by a process of cell competition. The role of CP in preventing tumorigenesis might be related to its major function, preventing excessive actin polimerization, or to additional functions, such as the maintenance of epithelial cell polarity.

The tumor suppressor function of CP appears to be tissue specific since the above cell behavior is only observed in restricted epithelia. This suggests that each epithelium has specific cytoskeleton and/or junctional properties, making cells sensitive or not to mutations that cause abnormal tumor growth. Altogether the presented data highlight the crucial impact of tissue context for the activation of a tumoral process.

Keywords: Capping Protein, epithelium to mesenchymal transition, epithelia and tumor.

RESUMO

O citosqueleto de actina desempenha um papel fundamental no controlo da forma e mobilidade celulares. Dada a sua dinâmica, é necessária uma regulação eficaz, que é desempenhada por proteínas que se ligam à actina. As proteínas *Capping protein a* (Cpa) and *Capping protein ß* (Cpb) são duas subunidades do heterodímero *Capping Protein* (CP) que se liga ao filamento de actina, prevenindo a adição de monómeros e, desta forma, a sua polimerização excessiva.

Nos tecidos epiteliais, a região apical das células contém uma banda de filamentos de actina, essencial para o suporte das junções celulares. Quando ocorre perda de adesão num epitélio, as células podem sofrer uma transição epitélio-mesenquimatosa, durante a qual se tornam resistentes aos mecanismos de controlo de crescimento dos tecidos e à morte celular e adquirem potencial migratório. Estas alterações celulares caracterizam a progressão de tumores malignos.

Curiosamente, quando clones de células mutantes para ambas as subunidades do heterodímero *CP* são induzidos no tecido selvagem heterozigota do disco imaginal da asa de *Drosophila*, as células mutantes na região dorso-ventral (DV) do disco, designado por zona do *wing blade*, sofrem extrusão basal e morrem por apoptose. Contudo, no epitélio do foliculo ovárico de *Drosophila*, a indução de clones de células mutantes para o gene *cpa* leva a um tipo de comportamento celular diferente: as células perdem as suas características epiteliais, sobre-proliferam e parecem adquirir potencial migratório, capaz de invadir tecidos ajdacentes. Este comportamento diferencial assemelha-se ao comportamento de células mutantes para genes supressores de tumores neoplásticos em *Drosophila*. Nestes casos, pequenos grupos de células mutantes são removidos pelo tecido adjacente, que lhes emite um sinal de morte celular. Este mecanismo designa-se por competição celular e é considerado crucial para a supressão de tumores.

Desta forma, o presente trabalho tinha como objectivo comprender de que forma o contexto celular pode influenciar o comportamento das células mutantes para ambas as formas do heterodímero CP, levando a fenótipos completamente diferentes. Pretendia ainda, de forma inerente, avaliar o potencial supressor de tumores do heterodímero CP. Assim, dado que as mutações para os genes *cpa* e *cpb* são letais, usei a técnica de ARN

de interferência (RNAi) para induzir o silenciamento pós-transcricional de ambos os genes. As técnicas disponíveis em *Drosophila* (sistema UAS-Gal4) permitiram-me direccionar o silenciamento de *cpa* e *cpb* para regiões específicas do seu tecido, de forma a poder avaliar a influência do contexto do tecido no comportamento de células silenciadas para os genes *cpa* e *cpb*.

Quando o gene *cpb* foi silenciado em toda a região do *wing blade*, a maioria das células desta região sobreviveram e pareciam ter perdido o controlo da proliferação celular bem como a polaridade epitelial comprometida. A sua morfolgia parecia também ter-se alterado, tornando-se mais arredondada e menos adesiva. Todas estas observações sugerem que estas células poderiam estar a sofrer uma transição do estado epitelial para o estado mesenquimatoso. O comportamento diferencial entre a inactivação de *cpb* por indução de clones e por silenciamento através de RNAi poderia ocorrer por uma questão de dosagem proteica, já que em clones os níveis de *cpb* são nulos e o RNAi leva apenas a um silenciamento parcial da expressão génica. No entanto, este não parece ser o caso, já que o silenciamento por RNAi em regiões restritas das asa levou à extrusão epitelial e morte celular. Desta forma, a extensão da inactivação de *cpb* parece ser determinante para o fenótipo celular: o silenciamento de *cpb* numa vasta região do disco imaginal da asa remove a contribuição do tecido do tipo selvagem, logo não há sinalização de morte celular para as células adjacentes e estas sobrevivem. Considera-se assim, que o contexto celular é determinante para a sobrevivência de células cujo desenvolvimento é anormal.

Para além de permitirem explicar o fenótipo de clones de células mutantes para *CP*, os resultados obtidos quando o gene *cpb* foi silenciado em toda a região do *wing blade*, sugerem que o heterodímero *CP* pode funcionar como supressor de tumores. Desta forma, são apresentadas no presente trabalho as diferentes hipóteses que podem explicar o papel do heterodímero *CP* neste processo. Em primeiro lugar propõe-se que a função principal de CP, de prevenir a polimerização excessiva dos filamentos de actina, possa prevenir a formação de tumores. Isto seria explicado pelo facto do citosqueleto de actina estar envolvido numa multiplicidade de fenómenos celulares, cuja desregulação pode levar à activação de processos tumorais. Um exemplo é o seu papel no tráfego vesicular, ou, em alternativa, a existência de isoformas especificas de actina localizadas preferencialmente em determinados epitélios, cuja acumulação poderia activar tumores.

O heterodímero CP localiza-se apicalmente nos tecidos epiteliais e estudos anteriores sugerem que este possa estabelecer a ligação dos filamentos de actina à membrana celular. Desta forma, propõe-se que este possa ter um papel no estabelecimento ou manutenção da polaridade epitelial, cuja destruição é crucial para a activação tumoral. Uma vez que o fenótipo de perda de função hipomórfica de *cpb* e do gene *arc*, envolvido na modulação das junções de aderência são semelhantes, postula-se que ambos os genes possam interagir de forma cooperativa neste processo.

Adianta-se ainda uma hipótese concreta através da qual a activação de tumores poderia ocorrer. Esta baseia-se no facto do fenótipo de perda de função de *cpb*, tanto numa extensão reduzida ou em larga extensão, ser semelhante ao de perda de função dos genes *csk* e *dASPP*, ambos inibidores da família de proto-oncogenes Src. Sabe-se que o aumento dos níveis de actividade de Src leva à dissolução das junções de aderência e adesões focais, levando à formação de tumores. Desta forma, considera-se como hipótese que o heterodímero CP possa ser necessário para manter o nível de actividade de Src, prevenindo desta forma a formação de tumores. Esta hipótese é consistente com o facto de ambas as proteínas se localizarem apicalmente, possivelmente co-localizando nas junções de aderência. A hipótese de sobreactivação do proto-oncogene Src não exclui as duas hipóteses mencionadas anteriormente, já que pode surgir como uma consequência secundária de defeitos no citosqueleto de actina ou de defeitos de polaridade epitelial, causados directamente por perda de CP.

Curiosamente, o possível potencial suppressor de tumores de CP parece ser específico de determinados tipos de tecido, nomeadamente da região do *wing blade* do disco imaginal da asa e do tecido epitelial do ovário. Isto sugere que a arquitectura do citosqueleto e/ou das junções epiteliais é específica de cada epitélio, tornando as células mais ou menos sensíveis a mutações que causam desenvolvimento de tumores. No conjunto, os dados apresentados apontam para o impacto crucial do contexto celular na activação de um processo tumoral.

Palavras-chave: *Capping protein*, transição epitélio-mesenquimatosa, epitélio e tumor.

TABLE OF CONTENTS

I. INTRODUCTION

1. Molecular mechanisms of epithelial architecture

1.1 Epithelial structure

Most animal tissues are composed of epithelial sheets, which serve two main functions: protection from the external environment and separation of two different chemical milieus. An epithelium consists of a laterally coherent sheet of cells with distinct apical-basal polarity. Several types of junctions mediate cell-cell and cell-matrix contact in epithelia. The most relevant junctions for adhesion are the adherens junctions (AJ) and hemiadherens junctions (Schock and Perrimon, 2002).

The AJ is a generally circumferential junction located just basal to the apico-basolateral boundary, which major function is to link the actin cytoskeleton of neighboring epithelial cells (Fig. 1). They are composed of the transmembrane protein E-cadherin, which is linked on its cytoplasmic side to ß- catenin (in *Drosophila* encoded by *armadillo*) and α- catenin. In turn, α- catenin binds to actin filaments, forming a continuous band, the zonula adherens (ZA) (Tepass et al., 2001).

The hemiadherens junctions are spot-like junctions connecting the actin cytoskeleton of epithelial cells to the basement membrane. The primary function of basement membranes is to anchor down the epithelium to a loose connective sheet underneath but they also act as a mechanical barrier, preventing malignant cells from invading deeper tissues (LeBleu et al., 2007). The interaction between epithelial sheets and the basement membrane is mediated by integrins, which bind to components of the extracelullar matrix (ECM). Developing tissues contain focal adhesions (FA), which are the precursors of hemiadherens junctions. These structures confer strong adhesion to the substrate and are anchored to bundles of actin microfilaments, called actin stress fibers (Fig. 1). Stress fibers are mostly present in migrating cells and are required for generating the propulsive force for motility (Schock and Perrimon, 2002).

In addition to the adherens and hemiadherens junctions, three other junctional complexes exist in *Drosophila*: gap junctions, septate junctions (SJ) and the subapical region (SAR) (Fig. 1). Gap junctions form intercellular channels allowing the transmission of ions and small molecules between cells. In contrast, SJ act as

permeability barriers between cells. SJ contain a complex of three proteins, Discs large (Dlg), Lethal giant larvae (Lgl) and Scribble (Scrib) (Lgl complex), and locate apically to gap junctions but basal to AJ. SAR is the most apically localized junctional complex and it is defined by the accumulation of two protein complexes: the Crumbs (Cbr), Stardust (Std), Discs lost (Dlt) complex (Crb complex) and the Bazooka/Par-3 (Baz-Par-3), atypical Protein Kinase C (aPKC), Par-6 complex (Baz-Par3 complex) (Schock and Perrimon, 2002; Tepass et al., 2001).

Although the core molecular mechanisms regulating establishment and maintenance of the junctional complexes are similar in vertebrates and invertebrates, they operate within significantly different epithelial architecture. In vertebrates the most apical region of epithelial cells contains components of the TJ and in a basal position are located components of the AJ.

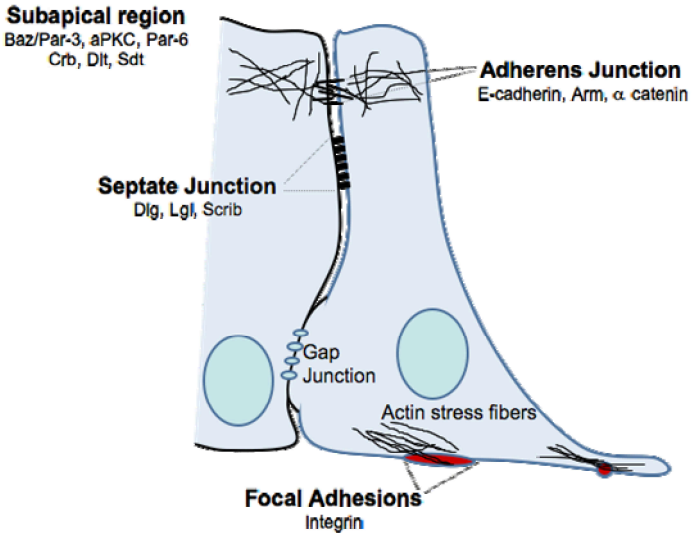


Fig. 1. Schematic representation of a *Drosophila* epithelial cell. Modified from Schock and Perrimon (2002). The major junctional complexes are represented: the apically localized subapical region, the adherens junction and the septate junction (whose molecular components regulate the establishment and maintenance of epithelial polarity), the gap junction and finally focal adhesions (precursors of hemiadherens junctions), which are involved in cell-matrix interactions.

1.2 Establishment and maintenance of epithelial structure

As mentioned, the molecular mechanisms involved in the establishment and maintenance of epithelial apicobasal polarity and formation of junctional complexes are highly evolutionary conserved between vertebrates and invertebrates. Since in my work I will use *Drosophila* epithelial cells, I will focus on the mechanisms described in this process for *Drosophila.*

In *Drosophila*, three sets of proteins located at defined positions along the apical-basal axis are responsible for the establishment and maintenance of polarity. These are

the SAR components, Crb and Baz/Par-3 complexes, and the Lgl complex. The first two protein groups are required for the formation of the ZA, which is the primary event for the establishment of epithelia structure. Whereas the Baz/Par-3 complex regulates early phases of ZA assembly, the Crb complex is required for later phases of ZA maturation. Crb protein is considered to be a crucial apical determinant, since it has the ability to confer apical membrane identity to the basolateral membrane. The Lgl complex components are located at the SJ, basal to the ZA and counteract Crb complex apicalizing activity (Gibson and Perrimon, 2003; Schock and Perrimon, 2002).

1.3 *Drosophila* as a model system to understand epithelial architecture

Since the main mechanisms governing epithelial polarity in vertebrates and invertebrates are highly conserved, *Drosophila* arises as an interesting model to study epithelial architecture (the great success of *Drosophila* as a model organism to study epithelial architecture relies on the advantages derived from its use, which are mentioned in attachment 1). Models for epithelial differentiation in postembryonic development of *Drosophila* include the imaginal discs and the ovarian follicle epithelium. While imaginal disc epithelia are preferred for the analysis of epithelial maintenance and specialization of epithelial surface domains, the follicular epithelium, due to its unique characteristic of renewing itself constantly, allows the analysis of the full range of phases in epithelial differentiation (Tepass et al., 2001).

Imaginal discs are larval structures that serve as the primordial for most adult structures. They are established during embryogenesis as clusters of 20-50 cells, but proliferate to a final size of 20000-50000 cells before they undergo metamorphosis. The imaginal disc epithelium is composed of a squamous layer, the peripodial membrane, and of a columnar layer of proliferating cells, the proper disc epithelium (Fig. 2 C) (Hariharan and Bilder, 2006). Wing imaginal discs are subdivided into three regions along the dorsoventral (DV) axis of the disc: the notum (or body wall), the hinge and the pouch (or blade) (Fig. 2 B). These regions are genetically defined during early larval development. An antagonist relationship between Epidermal Growth Factor (EGF) and Wingless (Wg) patterns the disc into the dorsal region which will give rise to the notum, and a ventral region which forms the wing. The wing is further subdivided by expression of selector

genes *vestigial* (*vg*) and *scalloped* (*sd*) in the wing blade and *homothorax* (*hth*) in the wing hinge (Klein, 2001). Vg is required for wing formation (Williams et al., 1991) and its misexpression can induce ectopic wing tissue (Kim et al., 1996).

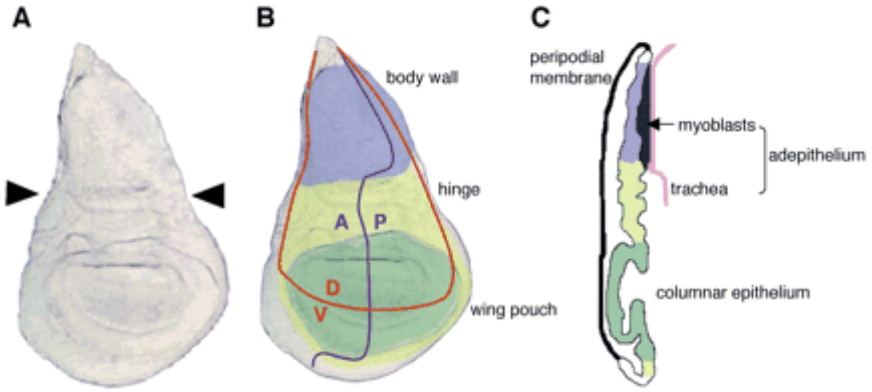


Fig 2. ***Drosophila* wing disc and fate maps.** Adapted from (Butler et al., 2003). A) Third instar wing imaginal disc. B) Fate map of the wing disc showing the anterior-posterior (AP) and dorsal-ventral (DV) compartment boundaries and major regions in the disc. In the adult, the wing pouch (green) gives rise to the wing, the hinge (yellow) constricts to form a mobile link to the body wall (blue) of the fly. C) Cell layers of the wing disc. There are two main cell layers: the peripodial membrane and the columnar epithelium that gives rise to the adult epidermis.

1.4 Remodeling of epithelia in normal and abnormal developmental processes

Cell phenotype transitions involving modulation of cell-cell adhesion occur during both physiological and pathological states. During embryogenesis of most animals, epithelial cell subpopulations actively downregulate cell adhesion systems and leave their local neighborhood to move into new microenvironments were they eventually differentiate into different cell types. This regulated phenotypic modulation is called epithelial to mesenchymal transition (EMT). Also in cancers, more precisely during the progression of primary tumors towards metastasis, tumor cells lose epithelial characteristics, change cell shape and acquire migratory abilities and invasiveness. Despite these different situations, the final effectors controlling cell phenotype, including the cytoskeleton, the cell-cell and the cell-matrix adhesion systems, appear to be similar or identical (Savagner, 2001; Huber et al., 2005).

Both normal and pathological versions of EMT involve, in addition to changes in shape and acquisition of motility, fundamental alterations in the gene expression profile. The expression of the adhesion component E-cadherin is repressed while the expression of vimentin, an intermediate filament component of the mesenchymal cell cytoskeleton is induced. Additionally, the cells start producing fibronectin and expressing N-cadherin. Moreover, there is increased expression of proteases, in particular metaloproteases, since the detachment of epithelial cells from the basement membrane requires their increased activity, as well as a dramatic reorganization of the actin cytoskeleton. This is an extremely important feature for the acquisition of motility and is thought to involve a complex regulation of the actin cytoskeleton effectors, in particular the small GTPases of the Rho family (Weinberg, 2007).

2. Tumor formation and development

Cancer is a multistep complex disease, resultant from genetic and epigenetic changes that occur in cells of various cell types. In mammalian epithelial cells, the activation of a neoplastic tumoral process is characterized by disruption of epithelial apicobasal polarization and acquisition of mesenchymal characteristics (EMT), self sufficiency in growth/proliferation signals (which leads to tissue overproliferation), resistance to cell death, inability to differentiate and ultimately, acquisition of migratory and invasive abilities (Hariharan and Bilder, 2006).

Two main categories of genes are involved in the process of carcinogenesis: oncogenes, which are the result of activated proto-oncogenes, and tumor suppressor genes (TSG). Both types of genes are required for normal cell proliferation and differentiation, and aberrant expression leads to abnormal cell proliferation.

Despite the differences in tumor development in mammalian systems and *Drosophila*, during recent years its use to understand tumorigenesis has become widely accepted. The late mechanisms of tumor development may be considerably different, since *Drosophila* has an open circulatory system, thus does not undergo angiogenesis, and has a different system of telomere maintenance, however, the basic cell biology mechanisms appear to be similar in both systems (Brumby and Richardson, 2005). Therefore, from now on, I will focus my analysis on *Drosophila* TSG and oncogenes.

2.1 Tumor supressor genes

TSG are genes that, when mutated to a loss of function, result in excessive tissue growth. The TSG affecting epithelial tissues may be classified as hyperplastic, when their inactivation leads to increased cell proliferation but normal tissue architecture is maintained or neoplastic, when in addition to increased proliferation there is disruption of epithelial structure. Neoplastic tumors are also characterized by an inability to terminally differentiate and acquisition of invasive characteristics, resembling mammalian malignant tumors (Bilder et al., 2000; Hariharan and Bilder, 2006).

Among the hyperplastic TSG identified until now, *pten*, *Tsc1*, *Tsc2*, *merlin*, *expanded* and components of the warts pathway are some of the most studied in *Drosophila*. Less characterized ones include the *C-terminal src kinase* (csk) and *discs overgrown* (*do*). The neoplastic TSG may be included in two major groups: genes of the endocytic pathway (*avalanche*, *rab5*, *tgs101* and *vps25*) and genes of the junctional scaffold (*scrib*, *dlg* and *lgl*) (Hariharan and Bilder, 2006).

The phenotypes of mutants for endocytic neoplastic TSG are caused by an excess accumulation of various signaling receptors that fail to be turned over in mutant tissue (Hariharan and Bilder, 2006). In particular, defects of *avalanche* and *Rab5* mutant cells appear to be due to mistrafficking of the polarizing component Crb (Lu and Bilder, 2005).

As mentioned, proteins of the Lgl group (Lgl/Dlg/Scrib) localize basolaterally, at the SJ. Bilder and Perrimon (2000) were the first to report a genetic interaction between these three neoplastic TSG, as well as their cooperative regulation of cell polarity and growth. Homozygous larvae for any of these genes undergo an abnormal third instar larvae period of two weeks and fail to pupate. During this period, imaginal discs and brain cells over proliferate in a disorganized pattern, forming large amorphous masses. Mutations in these genes also display invasive characteristics, particularly seen in the ovarium follicle epithelia. Unlike the imaginal disc epithelia, the follicle epithelium is in close contact with a distinct tissue, the germ line, and is enclosed in a monolayered sheath. Mutant cells for components of the Lgl complex are capable of leaving the epithelium and streaming into the germ line cyst, where they penetrate between nurse cell membranes (Fig. 3). Interestingly, despite the continuous proliferative ability acquired by

mutant cells, the proliferation rates are slower in mutant than wild type cells (Humbert et al., 2003). When clones of mutant cells for components of the Lgl complex are induced through the process of mitotic recombination, the wild-type neighboring cells ultimately eliminate clones by a phenomenon known as cell competition (Bilder et al., 2000), showing that the surrounding wild type tissue may account to the suppression of a tumoral process. Inded, when *scrib* clones in mosaic eye discs are protected from the cell competition events, mutant cells overgrow and lead to host lethality (Brumby and Richardson, 2003).

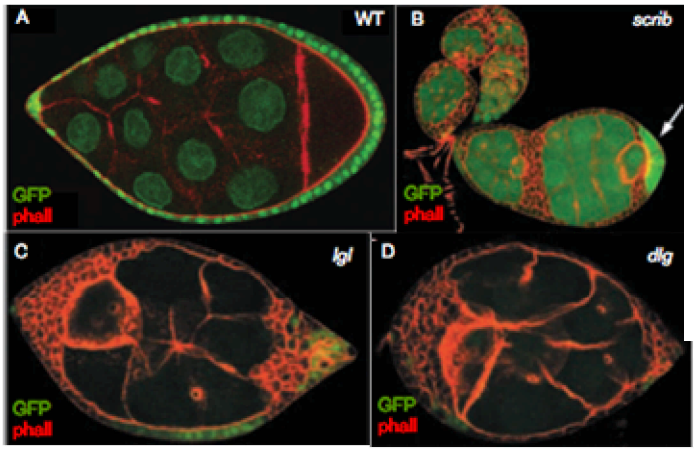


Fig. 3 Groups of cells homozygous mutant for genes of the Lgl complex overproliferate and show an invasive type of behavior in the ovarium follicle epithelium. Adapted from Bilder and Perrimon (2000). Mutant cells are marked by the absence of GFP. Phalloidin marks actin filaments.

Another TSG that was reported to be highly susceptible to the surrounding wild type tissue is *csk* (Vidal et al., 2006). As previously mentioned, *csk* is classified as a hyperplastic TSG. Homozygous mutants die during pupal stage and their imaginal discs are up to 50% larger than wild type discs. Csk is known to negatively regulate the kinase activity of the oncongenic family of the Src Kinases, thus *csk* inactivation is predicted to result in a Src increased activity (Yeatman, 2004). Interestingly, while broad loss of *csk* leads to enlarged, mispatterned tissues due to a block in apoptosis, overproliferation and decreased cadherin mediated adhesion, discrete inactivation of *csk* leads to epithelial exclusion, invasive migration and apoptotic death (Fig. 4) (Vidal et al., 2006). These differences in outcome highlight the relevance of tissue context on tumor growth.

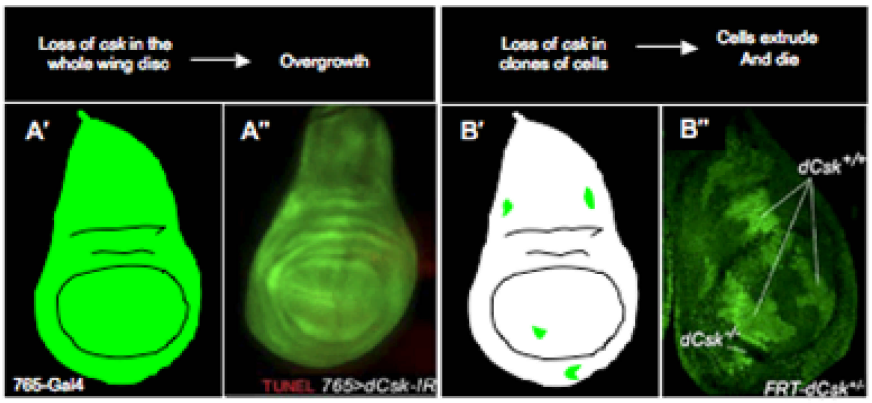


Fig 4. **Contrasting phenotypes caused by differential loss of *csk* in *Drosophila* wing imaginal disc.** Images modified from Vidal et al., 2006. A) Loss of *csk* in the entire wing disc leads to tissue overgrowth. Depletion of *csk* was induced through RNAi silencing and targeted to specific domains of *Drosophila* tissue through the use of the GAL4/UAS system. *csk* depletion was targeted to the entire wing disc using the 765-Gal4 driver, which domain of expression in shown in A'. In A'', is shown a third instar wing imaginal disc targeting depletion of *csk* in the entire wing disc. Those discs are enlarged. B) Loss of csk as discrete patches leads to epithelial extrusion and cell death. B' is a schematic of the mutant clones, while B'' shows that *csk* mutant cells are eliminated from the tissue.

2.2 Oncogenes

Oncongenes, like TSG may be classed as hyperplastic or neoplastic, according to the same criteria. Hyperplastic oncogenes include Src and the activated forms of Ras (Ras^{act}) and Notch ($Notch^{act}$). The neoplastic include the activated PDGF- and VEGF-receptor related (Pvr) (Brumby and Richardson, 2005).

Ras and Notch signaling are involved in a variety of cellular processes, which frequently overlap and may influence each other, either in a cooperative or antagonistic manner. Ras is a small GTPase that cycles between an inactive GDP-bound state and an active GTP-bound state. Activated Ras in *Drosophila* exerts its oncogenic effects through Raf/MAPK pathway, whose targets have been shown to promote differentiation, cell survival and cell proliferation (Sundaram, 2005). In turn, Notch consists of a transmembrane protein, which intracellular domain is implied in promoting nuclear transcription. Notch signaling pathway was reported to have both tumor suppressor and inducing functions during the process of tumorigenesis, but the mechanisms regulating this process in *Drosophila* are quite complex and are not completely understood (Brumby and Richardson, 2005; Weng and Aster, 2004).

The role of Src kinases in tumorigenesis is postulated to initially promote cell growth but it also regulates other cellular activities such as adhesion, motility and

migration during late stages of tumorigenesis, possibly due to different levels of activity (Vidal et al., 2007). Src localizes at AJ and FA, and its overactivity disrupts these two structures, therefore reducing adhesion. In the AJ, the Src kinase enhanced activity suppress E-cadherin localization and function, while in FA it binds to Focal Adhesion Kinase (FAK), activating it. FAK signaling ultimately activates the Jun N-terminal Kinase (JNK) signaling pathway, promoting expression of metaloproteases such as MMP3 and MMP9, which together with decreased E-Cadherin promote invasiveness (Yeatman, 2004).

2.3 Tumor microenvironment and the combinatory model

The development of cancer is a multistep process, which is thought to involve the cooperation of several mutations, as well as interactions between the tumor and its microenvironment. When clones of mutant cells for the TSG *scrib* and *csk* are induced among wild type tissue, the surrounding wild type cells actively remove the abnormal cells through the process of cell competition (Brumby and Richardson, 2003; Vidal et al., 2006). The molecular mechanisms that trigger cell competition are still not completely understood, however the proto-oncogenic family of Myc is postulated to mediate this process: clones of cells expressing low levels of Myc are out-competed by neighboring cells that express higher levels of Myc (Johnston et al., 1999). Also, the competition for survival factors such as decapentaplegic (DPP) could account for this effect (Moreno et al., 2002). Either induced by Myc or by Dpp, the JNK signaling pathway is thought to be the final effector of the cell competition process (Adachi-Yamada and O'Connor, 2004; Moreno et al., 2002).

Since wild type tissue has mechanisms of suppressing tumor development, mutations in a single gene may not be sufficient for the activation of a tumoral process and a cooperative interaction between TSG and oncogenes may be required for the formation of invasive type of tumors. As mentioned above, *scrib* mutant cells are not capable of competing with the surrounding wild type tissue and of behaving as inasive tumor cells. However, the combination of the activated alleles of either $Notch^{act}$ and Ras^{act} with clonal inactivation of *scrib*, results in a strong neoplastic growth, capable of invading distant tissues and metastasing (Brumby and Richardson, 2003; Pagliarini and

Xu, 2003). These experiments provided the basis to the establishment of the combinatory model in *Drosophila*. The combinatory model postulates that tumor development in flies, like in mammals is influenced by the tissue context, and additional alterations, either within the tumor or in the surrounding normal tissue, might be required to result in unrestrained tumor growth and lethality (Brumby and Richardson, 2005).

3. The actin cytoskeleton in normal and pathological developmental processes

3.1 The actin cytoskeleton and its dynamics

The actin cytoskeleton plays a central role in numerous cellular processes. It functions in the generation and maintenance of cell morphology and polarity, in endocytosis and intracellular trafficking, in contractility, motility and cell division. Actin is a globular, nucleotide-binding protein that is present in cells in two main forms, the globular (G) actin and the filamentous (F) actin. The filamentous actin is a polarized structure, in which the barbed or growing end is the preferred site for ATP-monomeric-actin addition, whereas the pointed or slow growing end is the predominant site for loss of ADP-actin subunits (Fig. 5). This ATP-hydrolysis-driven filament growth is a very dynamic process, which requires a tight regulation *in vivo*. More than 70 classes of Actin Binding Proteins (ABP) control the dynamics of actin filaments within the cell, regulating their assembly, disassembly and organization (dos Remedios et al., 2003; Winder and Ayscough, 2005).

ABP such as Profilin, Formins, Enabled/VASP (Ena/VASP) family proteins and the Arp2/3 complex promote actin polymerization. Profilin enhances the exchange of ADP for ATP on actin monomers, Formins as well as the Arp2/3 complex are important for *de novo* filament formation, a process known as nucleation. The Arp2/3 complex has also a role in branch formation and its activity is enhanced by the WASP and SCAR/WAVE proteins. In contrast, the Ena/VASP family proteins favour formation of long un-branched filaments. Among the ABP that prevent actin polymerization, the Actin Depolymerizing Factor (ADF)/Cofilin family, the Cyclase associated protein (CAP) and Capping Proteins (CP) are some of the most important (Fig. 5). The ADF/Cofilin severs filaments and enhances dissociation of actin monomers from the pointed end, CAP sequesters actin monomers, preventing their incorporation into filamens and CP restricts

accessibility of the barbed end, inhibiting adition or loss of actin monomers (dos Remedios et al., 2003; Winder and Ayscough, 2005).

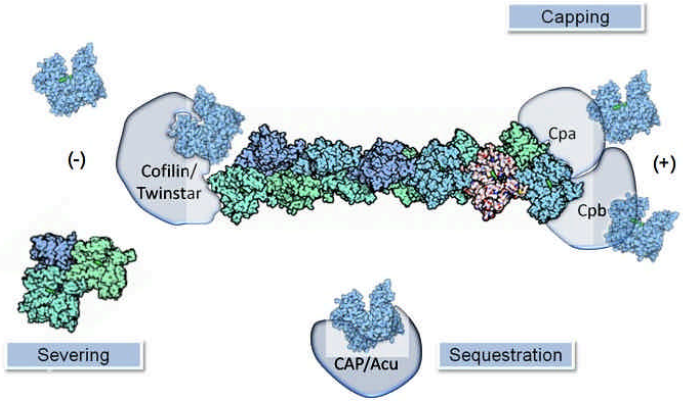


Fig. 5 Schematic representation of an actin filament and some of its most important regulators within the cell. All the ABP represented in the figure prevent excessive actin polymerization, but through different processes. CAP/Acu sequesters actin monomers, while Cofilin and Twinstar promote severing of the actin filament and CP binds to the barbed end of the filament, preventing addition or loss of actin monomers.

3.2 The actin cytoskeleton in epithelia

The polarized actin cytoskeleton is a key feature of epithelial cells. A continuous band of actin filaments localized apically links epithelial cells to each other, forming the ZA. This is extremely important for the maintenance of the epithelia, since it provides adhesive strength between epithelial cells. Also, other important functions of the actin cytoskeleton such as directed intracellular trafficking seem to require polarization of actin filaments within epithelial cells (Apodaca, 2001).

The polarization of actin filaments requires distinct mechanisms of actin regulation within the domains of the epithelial cell, however few studies have focused this issue. In 2001, Baum and Perrimon reported the role of CAP, Ena and Abl in the modulation of apical actin-filament formation, possibly by acting in concert. In contrast, Profilin and Coffilin seem to have a more global function, regulating cortical actin filaments dynamics throughout the whole cell. It has been also proposed that CP might play a role in linking the actin cytoskeleton to the membrane (Wear and Cooper, 2004).

3.3 The actin cytoskeleton in pathology

The actin microfilament system is considered to be the engine of cellular migration. Numerous actin effectors provide a temporally and spatially controlled turnover of actin structures, which is crucial to drive protrusion of migrating cells. Therefore, due to the complex regulation of the actin cytoskeleton, the system is vulnerable to mutations and defects that may cause a wide range of disorders. These may affect embryonic development, the immune system or lead to tumor invasion and metastasis, which characterize cancer malignancy (Lambrechts et al., 2004; Yamazaki et al., 2005).

Cancer is often a genetically heterogeneous disease. Although many mutations that are in the origin of tumorigenesis affect non-cytoskeletal proteins, the overall result is a transition from an immotile to a motile cell and an inherent dramatic alteration of the actin system. These apparently arise via three non-mutually exclusive pathways: mutations in actin, changes in the upstream regulatory proteins or changed expression levels of ABP. Among the upstream regulators that are known to be involved in this process, the Rho-GTPases are thought to play an important role, particularly through activation of the WASP family proteins (activators of the Arp2/3 complex) (Lambrechts et al., 2004).

Also, the expression levels of several ABP, such as beta-tymosins (monomer sequestering), Arp2/3, cortactin, WASP, profiling (filament nucleation and elongation), gelsolin, cofilin (filament capping, severing, or depolymerisation), have been reported to be altered during human tumors. However, this is probably not the primary cause of oncogenecity but rather an inherent consequence of the altered cellular background (Lambrechts et al., 2004; Yamazaki et al., 2005).

4. Capping protein

4.1 CP: a highly conserved heterodimer

CP is a highly conserved heterodimer, composed of capping proteins α (Cpa) and β (Cpb), which cap the barbed end of actin filaments with high affinity, thereby preventing excessive actin polimerization (Wear and Cooper, 2004). Several studies account for the role of CP in actin assembly and regulation of cell motility. In *Dictyostelium*, an increase in CP leads to an increase in the cell movement (Hug et al., 1995), while *in vitro* studies

have shown that CP and the Arp2/3 complex show a functional antagonism, essential in regulating motility (Pantaloni et al., 2000). Also during *Drosophila* bristle development, Cpb acts antagonistically to the Arp2/3 complex in promoting the formation of a transient subpopulation of filaments, called snarls, which are localized between bundles (Frank et al., 2006). Although the mechanism and regulation of CP have been studied, most of the available data results from *in vitro* biochemical studies and cells in culture. Therefore, it is crucial to understand the role of CP and its regulation within a tissue.

4.2 CP maintains cells within the wing disc epithelia

Clonal analysis in the *Drosophila* wing imaginal disc revealed a new feature of CP: they are required for the maintenance of cells within the wing blade epithelium. Mutant cells for either *cpa* or *cpb* in these region suffer basal extrusion and apoptosis (Fig. 6). Interestingly, the requirement for CP to maintain cells within the epithelia is restricted to the wing blade region, since cell extrusion and death caused by loss of CP occurred in the blade but not in the hinge and notum regions (Janody and Treisman, 2006).

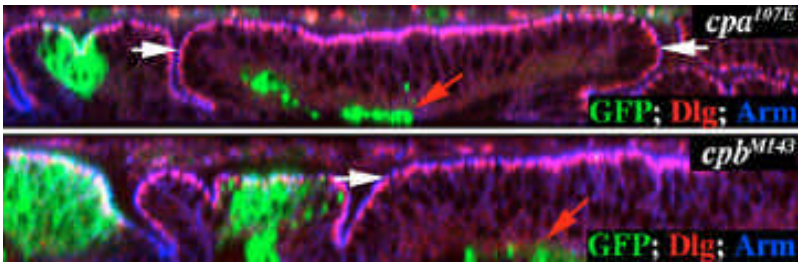


Fig. 6. Clones of mutant cells for *cpa* and *cpb* extrude from the wing blade epithelia. Adapted from Janody and Treisman (2006). Optical cross sections through the wing disc epithelium. Mutant clones are positively labeled with GFP (green) and discs are stained with anti-Dlg (red) and anti-Arm (blue) to outline apical cell membranes. Either *cpa* and *cpb* mutant clones are extruded from the wing blade epithelium (indicated by white arrows) but are maintained within the remainder regions of the disc.

4.3 CP prevents excessive actin filament polimerization and localizes at apical sites

As previously proposed by *in vitro* studies, CP prevents excessive actin filament polymerization in the *Drosophila* wing disc. However, it was shown that there is a cell type-dependent effect on actin filament accumulation in *cpa* mutant cells: in the notum and hinge region actin filaments accumulate at the apical region of the cell whereas in the blade, the accumulation occurs around the entire cell cortex (Fig. 7). However, excessive

actin filament accumulation may not be the first cause of extrusion of *cpa* mutant cells, since mutant cells for other ABPs that prevent excessive actin polimerization, such as Twinstar (Tsr) and Capulet (Capt), do not cause cell extrusion (Janody and Treisman, 2006).

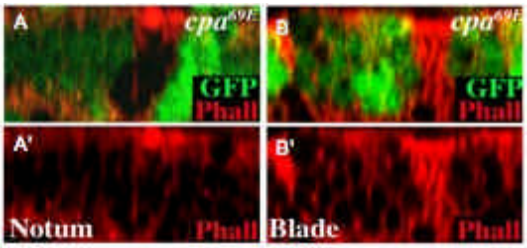


Fig. 7. Loss of cpa leads to cell-type dependent accumulation of actin filaments in the different regions of the wing disc. Adapted from Janody and Treisman (2006). A-B) Optical cross sections through third instar *Drosophila* wing imaginal discs, comparing the notum and blade regions, respectively. Clones are negatively marked by GFP (green) (A,B) and discs are stained with phalloidin to reveal F-actin (red) (A',B'). In the notum actin filament accumulation is restricted to the apical domains, while in the blade actin filaments accumulate throughout the cell.

By expressing a HA-tagged form of Cpa, it was observed that Cpa mostly accumulates at the apical regions of the cell, co-localizing with components of epithelial junctions (Fig. 8 A-B). Moreover, AJ components were shown to be mislocalized at basolateral positions in *cpa* mutant cells (Fig. 8 C). These observations show that there is a region specific requirement for CP to maintain apicobasal polarity in wing blade cells (Janody and Treisman, 2006).

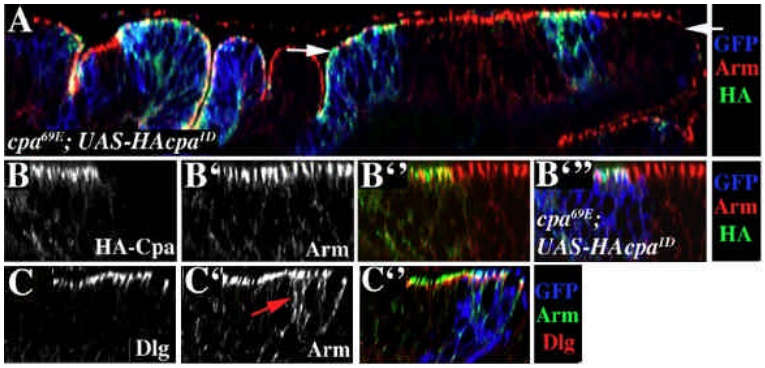


Fig 8. Cpa possibly localizes at apical membrane sites and maintains the localization of AJ components. Adapted from Janody and Treisman (2006). Optical cross sections through third instar *Drosophila* wing imaginal disc. A-B) An HA-tagged form of Cpa (green) rescues the cell extrusion phenotype in cpa mutant cells (blue) and co-localizes with the AJ component Arm (red). C) In *cpa* mutant cells (blue) the AJ components Arm (green) and Dlg (red) are mislocalized (indicated by a arrow in C').

4.4 The behavior of *CP* mutant cells depends on the Vestigial transcription factor

Cpa was shown to be required for the survival and maintenance of *vg*- expressing cells in the wing disc epithelium: *cpa* mutant clones misexpressing *vg* were maintained in all the regions of the wing epithelia and ectopic expression resulted in apoptotic death and extrusion in the entire disc. Furthermore, the expression levels of *cpa* were enhanced in the wing blade primordial, due to the presence of Vg. Thus, it is proposed that Vg alters the cytoskeletal structure of cells by upregulating the expression of *CP*, which leads to different cellular outcomes (Janody and Treisman, 2006).

4.5 CP maintains the monolayer character of the ovarian follicle epithelium

Clonal analysis in another type of epithelia, the ovarium follicle epithelia, leads to a completely different cellular outcome: *cpa* mutant cells lose their monolayer character, form disorganized multiple layers, leave the epithelium and stream into the germ-line cyst, where they penetrate between nurse the cell membrane (Fig. 9) (Janody, un-published data). These observations suggest that *CP* mutant cells might aquire an invasive cell behaviour, similar to neoplastic TSG mutant cells such as *scrib*.

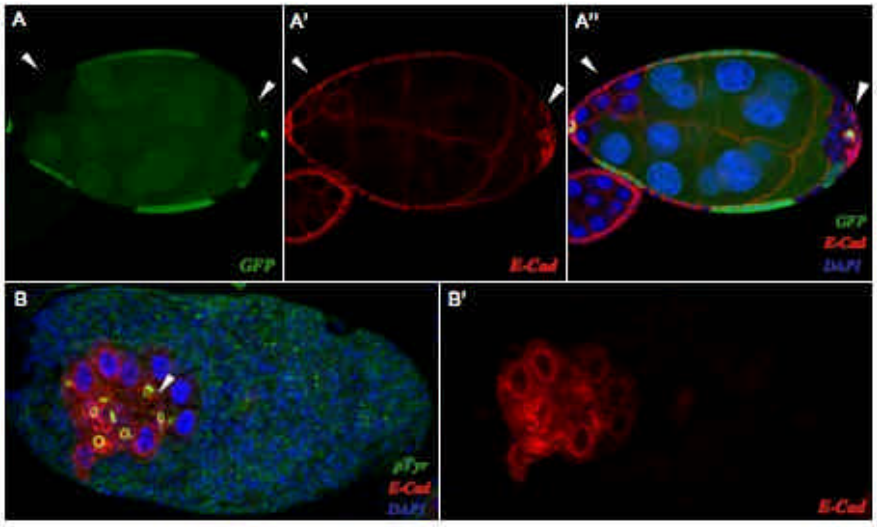


Fig. 9. Cpa maintains epithelial monolayer character of the ovarium follicle epithelia and prevents migratory type of behavior. Images obtained by Janody. Sections of the *Drosophila* ovaries. A) *cpa* mutant clones are negatively marked by GFP and indicated by arrows. E-Cad staining (red) and DAP1 (blue) show that cells lose epithelial architecture and overproliferate. B) DAP1 staining indicates *cpa* mutant cells of the ovarian follicle overproliferate and become invasive, since some are contained within the germ line cyst (indicated by arrow in B).

II OBJECTIVES

The Capping Protein α β heterodimer (CP) prevents epithelial to mesenchymal like transitions in restricted epithelia: in the *Drosophila* wing disc, only mutant cells located in the blade region extrude from the epithelium and die. In a similar manner, loss of either *csk* or the TSG *scrib* in discrete patches leads to cell extrusion and apoptotic death. However, broad loss of *csk* or *scrib* leads to a different outcome: enlarged and mispatterned tissues due to overproliferation and block in apoptosis (Brumby and Richardson, 2003; Vidal et al., 2006). This shows that the local environment influences the behavior of *csk* and *scrib*-deficient cells. Since in the follicle epithelium, groups of cells mutant for *cpa* form multiple layers, seem to overproliferate and are observed invading between nurse cells (see Fig. 9 of Introduction), resembling cells mutant for neoplastic TSG, the cell extrusion and cell death phenotype observed by clonal analysis of *CP* could be dependant on the surrounding tissue rather than caused by cellular defects (autonomous type of cell death). Therefore, the main objective of this work was to understand whether the cellular environment influences the behavior of *CP* mutant cells. Inherent to this, the work aimed to evaluate the potential of *CP* to suppress tumor formation in the wing blade, as occurs in the ovarium follicle epithelium. This attempted to show that epithelia may differ within the various type of tissues and that the tissue context may be crucial to the development of tumoral behavior.

III MATERIALS AND METHODS

1. Generation and establishment of the UAS-*CP*-IR transgenic lines

1.1 Generation of the UAS-*cpa*-IR and UAS-*cpb*-IR constructs

1.1.1 The pWIZ vector

The presence of double stranded RNA (dsRNA) causes the sequence-specific posttranscriptional silencing of a corresponding gene, thus this is considered to be an efficient method to inactivate genes of interest and study gene function. Although injection of dsRNA into *Drosophila* embryos is possible and effective, the effect is transient and is not inherited in the next generation. For that reason, methods have been developed to express dsRNA stably in transgenic *Drosophila* (Hannon, 2003; (Lee and Carthew, 2003)

The pWIZ vector was specifically designed to express dsRNA in *Drosophila*. As most of the methods, the pWIZ employs transgenes having an inverted repeat (IR) configuration, which are able to produce dsRNA as extended hairpin RNA. However, it differs from the other vectors due to the presence of a spacer between the inverted repeats. The spacer used is the 74-nucleotide second intron of the *white Drosophila* gene, which was shown to be efficiently spliced in heterologous tissues. This helps to stabilize recombinant plasmids, making the process of cloning easier (Lee and Carthew, 2003).

The pWIZ vector takes advantage of two very useful techniques in *Drosophila*: p-element mediated transformation and the modular Gal4/UAS expression system (described in attachment 1). Gene fragments are inserted in opposite orientations on each side of the intron, which is flanked by several unique restriction enzymes sites. The entire cassette is downstream of the UAS enhancer-promoter and upstream of the SV4 transcription termination site (Lee and Carthew, 2003). The overall procedure for making the *cpa* and *cpb* RNAi constructs in the pWIZ vector is described in Fig.1.

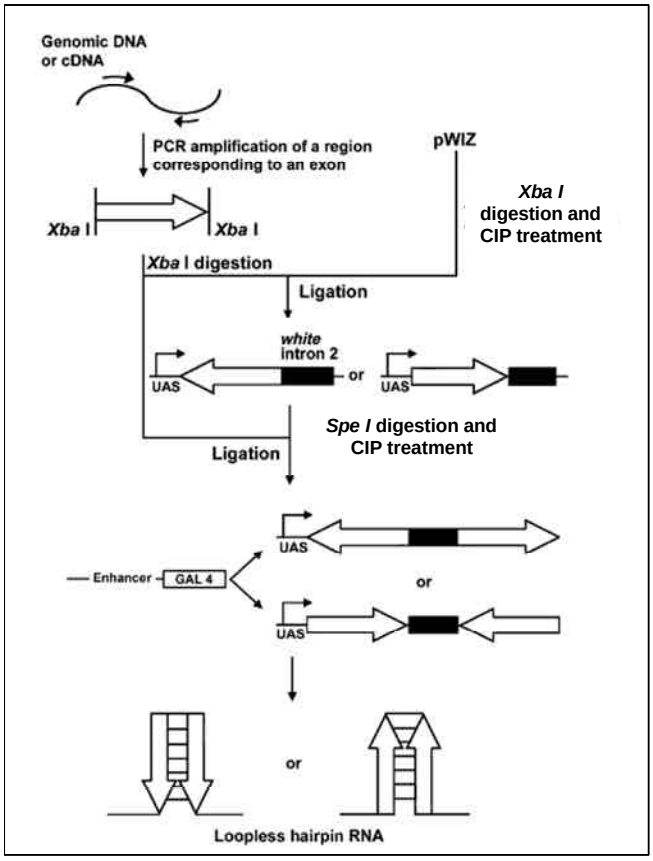


Fig. 1. Strategy of cloning used to generate the UAS-*CP*-constructs. Modified from Lee and Carthew (2000). The DNA fragment corresponding to *cpa* and *cpb* genes was amplified by PCR. A restriction site for XbaI was included in each PCR primer. The fragments and the pWIZ were digested with XbaI and ligated. This is the first cloning step into the pWIZ vector. The second cloning step occurs through digestion of the pWIZ vector containing the first insert with SpeI, whose free ends are compatible with the XbaI ones, and posterior ligation with XbaI-digested *cpa* and *cpb*. The gene fragments must be cloned in opposite orientations on each side of the *white* intron (IR), so that the expression of the IR produces hairpin-loop RNA that is competent to induce RNAi in *Drosophila*.

1.1.2 Design and amplification of the *cpa* and *cpb* IR

The choice of the gene fragments to clone in the pWIZ vector was performed according to several criteria: the fragment chosen should be 500 to 700 base pairs (bp); should correspond to an exon; should not have sequences in either sense and antisense orientation that match a 5' or 3' consensus splice site and should not have internal restriction sites corresponding to the primer sites (Lee and Carthew, 2003). Finally, a sequence of 443 bp for *cpa* and of 543 bp for *cpb* was chosen (attachment 2).

The gene fragments were amplified by polymerase chain reaction (PCR). The primers designed for each gene are shown in Tab. 1. In order to amplify the *cpa* gene fragment, the template used was the vector GH10050, which was obtained from Berkeley *Drosophila* Genome Project (BDGP). The *cpb* fragment was amplified from genomic DNA of WT flies.

Isolation of *Drosophila* DNA

In order to isolate the DNA of WT Drosophila, I anesthesized 20 flies and put them in an eppendorf. They were kept on ice until addition of 250μl of a solution containing 0,1M Tris-HCl, pH 9; 0,1M EDTA; 1% SDS and 0,5-1% DEPC. The flies were homogeneized with a 3mm plastic rod and incubated at 70ºC for 30 min. After incubation, 35 μl of Potassium Acetate 8M was added and the eppendorf was kept on ice for 30min. The solution was spinned at 4ºC for 15 min and the supernatant was transferred into a new tube. The DNA was purified by the phenol chlorophorm method (2:1) (described in attachment 3) and precipitated by addition of 0,5 volumes of isopropanol and centrifugation for 5 min at room temperature (RT).

Tab. 1. Description of the primers used to amplify the *cpa* and *cpb* regions to use as IR.

Gene	primer	Sequence (5'→ 3')	Characteristics
cpa	dscpb5'	ATTATCTAGA**GGTACC**AGCTCTGTTTTTGGAAAGGC	XbaI and **KpnI** restriction sites
	dscpa3'	ATTATCTAGATTATTGCGTCTTCAGTTCCT	XbaI restriction site
cpb	dscpb5'	ATTATCTAGA**GGTACC**TCGGAAATGCAGATGGACT	XbaI and **KpnI** restriction sites
	dscpa3'	ATTATCTAGAACCCTGTTTGTTGGTCTGC	XbaI restriction site

PCR conditions

The PCR had an initial 2 min denaturating cycle at 95ºC, which was followed by 35 cycles that included 1 min denaturation at 95ºC, 1 min of annealing at 58ºC and 1 min of polymerization at 72ºC. After the 35 cycles, an extension cycle of 5 min at 72ºC was included.

Confirmation and purification of amplified sequences

The PCR products were analysed by electrophoresis through an agarose gel (described in attachment 3). The purification of PCR products was performed using the NucleoSpin®Extract II (Macherey-Nagel), according to the kit instructions (attachment 3).

1.1.3 Cloning the *cpa* and *cpb* IR in the pWIZ vector

As described above, in Fig. 1, cloning the IR in the pWIZ vector is a two-step process.

1.1.3.1 First cloning step

Digestion with Xba I

The *cpa* and *cpb* gene fragments and the pWIZ vector were digested with XbaI (Takara) for 1h at 37ºC and then purified by the phenol choloform (2:1) method.

pWIZ phosporilation treatment

To prevent circularization of the plasmid, Shrimp Alkaline Phosphatase (S.A.P from Roche) was added to the previous solution. The reaction was performed at 37ºC for 30 min and the DNA was purified by the phenol chloroform (2:1) method.

Ligation

In order to ligate the plasmid and the insert, 100 ng of pWIZ were mixed with 16 ng of insert (which corresponds to a proportion of 1 plasmid to 3 inserts) and 0,25 µl of T4 DNA ligase (Promega). The reaction was performed at room RT for 3h.

Transformation

After the 3h of ligation, the volume of the reaction was added to 100 µl of DH5a cells. The eppendrofs were kept on ice for 20 min and transferred to an incubator at 42ºC for 2 min. After 2 min on ice, the transformed cells were added to 900 µl of LB medium. After 1 h incubation with agitation at 37ºC, the cells were plated in plates containing ampicilin and were left in an incubator at 37ºC overnight.

Purification of the plasmidic DNA

For each gene, 10-15 bacterial colonies were randomly chosen and put to grow in LB medium containing 50 µg/ml of ampicilin at 37ºC with agitation, overnight. 1,5 ml of bacterial culture was spinned for 2 min and the pellet was discarded. 300 µl of TS (Tris 50mM pH 7,5; Sucrose 25%) were added to the pellet and mixed. The same occurred with ELT (EDTA 100mM; Lysosyme 2 mg/ml; TritonX 100 0,1%). The mixture was incubated first at RT for 10 min and after, at 70ºC for 10 min. It was spinned at 4ºC during 15 min and the pellet was removed with a toothpick. 500 µl of PEG6000 20%; NaCl 1M solution was mixed with the supernatant and incubated for 30 min at RT.

Finally, the mixture was spinned for 4 min at RT, the supernatant discarded and the pellet was ressuspended in 45 µl TE.

Confirmation of the insertion

Each independent plasmidic DNA was digested with XbaI (Takara) for 1h at 37ºC and then run in an electrophoresis gel. The constructs which contained the insert were selected to perform the next cloning step.

1.1.3.2 Second cloning step

Digestion with Spe I

The selected constructs with the first insert cloned were digested with SpeI (Promega) for 1h at 37ºC and purified by the phenol chloroform (2:1) method.

The ligation, transformation and purification procedures are the same as the ones described in the first cloning step.

Confirmation of the insertion

Each insert contains a Kpn I restriction site to enable the selection of constructs with the correct orientation. Each independent plasmidic DNA was digested with KpnI (Takara) for 1h at 37ºC. The digestions were run in an electrophoresis gel and the plasmids which had both the inserts in inverted orientations were chosen and storen.

1.2 Establishment of UAS-*CP*-IR transgenic lines

1.2.1 Microinjection

In order to generate transgenic Drosophila lines containing the UAS-*CP*-IR constructs, I first prepared an injection mix. The injection mix contained the pWIZ with the IR for *cpa* or *cpb*, a helper plasmid containing a transposase gene (pTURBO) and water. The proportions of the mixtures prepared for each gene are represented in Tab. 2.

Tab. 2. Mixture used to *inject Drosophila* embryos.

	cpa	*cpb*
pWIZ+IR	22,25µl (300ng)	20,25µl (600ng)
pTURBO	2,68µl (75ng)	4,46µl (125ng)
water	4,8µl	5,29

The mixture was injected in W118 flies, which are *white*-. The flies layed for 45 min in plates containing apple juice and yeast and embryos were collected and dechorionated in commercial bleach till the appendages were lost. The embryos were aligned in an agarose gel and transfered to a cover slide, where they were stick with a mixture consisting of adhesive tape dissolved with heptane. Following dehydration by contact with sílica gel from 7 to 9 min, embryos were covered with Voltalef oil (10S), which allows embryos to breath and maintains hydration. The needles used for microinjection were pulled on a horizontal puller of the Sutter Instrument Co. Model p.97 and the microinjector used was from WPI, model pv 820. After microinjection, the embryos were kept in voltalef oil at 25ºC until larval stage, during which were transferred to vials with fly food.

1.2.2 Establishment of the RNAi stocks

The flies that were able to survive from the microinjection procedure are not transgenic, since the transgene was inserted in the polar cells of the embryo, which give rise to the germ line (see the principles of p-element mediated transformation in attachment 1). Thus, the surviving flies were backcrossed with W118 and the transgenic flies were identified within the progeny through the eye color (pW+). In order to establish a clean stock of the UAS-*cpa*-IR and UAS-*cpb*-IR, the transgenic flies were backcrossed with flies from the W118 stock. The mapping of the insertions is described in the results.

1. Fly husbandry

Flies were raised at 25ºC, under standard conditions (Roberts, 1998). The UAS-*CP*-IR lines were submitted to different temperature conditions to test the efficiency of the silencing effect (described in results).

Apart from the UAS-*CP*-IR transgenic lines I generated, several stocks of flies were used, which are described in Tab. 3. In addition the ones included in Tab. 3, I used a stock of W118 to generate transgene insertions: w-; *sco* / *cyo* ; *MKRS* / *TM6b*

Tab. 3. *Drosophila* stocks used. A) Stocks of transgenic flies used to direct expression, using the UAS-Gal4 system. B) Stocks of mutant alleles. Although I didn't use these lines, they are included because they had been previously recombined with the *vg*-Gal4 driver to enhance the RNAi silencing effect (described in results).

a)

	Line	Transgene location
UAS lines	UAS-*cpb*	3rd
	UAS-*dicer2*	X
	UAS-*CD8GFP*	3rd
Gal4 drivers	*vg*-Gal4	2nd
	sd-Gal4	X
	ptc-Gal4	2nd
	ey-Gal4	3rd
	da-Gal4	3rd
	30A-Gal4	2nd

b)

	Line	Type of mutation
Mutant alleles	cpa^{107E}	null
	cpb^{M143}	null

3. Immunohistochemistry

Third instar larvae wing imaginal discs were dissected in 0,1 M phosphate buffer (pH 7.2) and fixed in 4% Formaldehyde in PEM (0,1M PIPES pH 7,0; 2mM$MgSO_4$; 1mM EGTA) for 30 min on ice. The discs were washed in 0,1 M Phosphate buffer 0,2% Triton for 15 min on ice. The discs were incubated in primary antibody diluted in 0,1 m phosphate buffer, 0,2% triton, 10% serum, overnight, at 4ºC. The primary antibodies used were mouse anti-Armadillo (N27A1, 1:10; Developmental Studies Hybridoma Bank) and rabbit anti-Caspase 3 (1:500; BD Bioscience). After washing the discs in 0,1 M

phosphate buffer, 0,2 Triton, discs were incubated in secondary antibody for 2 h at 4ºC. Secondary antibodies were anti-mouse and anti-rabbit, from Jackson Immunoresearch, used at 1:2000, conjugated to TRITC and Cy5. Fluorescence images were obtained on a Zeiss LSM 510 META confocal microscope.

IV RESULTS

The Cellular environment influences the behaviour of *CP* mutant cells

In order to determine whether the cellular environment influences the behavior of *CP* mutant cells, I analyzed the consequences of *CP* depletion in restricted domains of the wing epithelium tissue. Since *cpa* and *cpb* mutant animals die as first instar larvae (Janody and Treisman, 2006), I made use of the RNA interference (RNAi) technology (Hannon, 2003) to target either *cpa* or *cpb* to degradation. I generated independent transgenic fly lines carrying either a UAS-*cpa*-IR or a UAS-*cpb*-IR construct under the control of the Gal4-responsive UAS enhancer repeats. Since the UAS-Gal4 system permits the temporal and spatial control of gene expression, expression of each UAS-*CP*-IR is controlled in time and space by the use of various Gal4 Drivers.

1. RNA interference as a tool to investigate the role of the cellular environment on the behavior of *CP* mutant cells

1.1 UAS-*cpa*-IR and UAS-*cpb*-IR transgenic lines

The design of the UAS-*cpa*-IR and UAS-*cpb*-IR constructs as well as the process of generation of transgenic *Drosophila* lines is described in Matherials and Methods. Three independent lines of UAS-*cpa*-IR and four independent lines of UAS-*cpb*-IR were obtained through p-element mediated transformation (Tab. 1). Since the P transposable elements (which contain the UAS-*CP*-IR constructs) integrate at random positions on the *Drosophila* genome, it is crucial to map the local of the transgene insertion to perform genetic analysis. Thus, both UAS-*CP*-IR transgenic lines were crossed with double balanced lines, and the locations for each insertion were determined and are shown in Tab. 1. The strategy used to map chromosome location of the lines is described in Fig. 1.

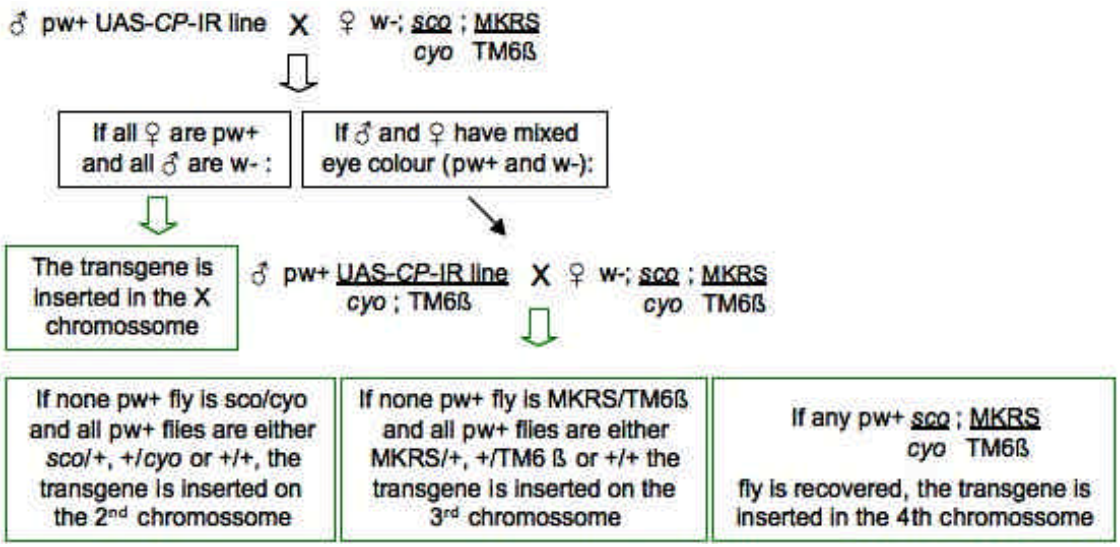


Fig 1. Schematic representation of the mapping procedure used to identify the chromosome location of the UAS-cpa-IR and UAS-cpb-IR constructs. For further information, see the principles of p-element mediated transformation, described in attachment 1.

1.2 Efficiency of the *UAS-CP-RI*

The use of the RNAi technique usually causes a drastic decrease in the expression of the target gene. However, since RNAi may not completely abolish gene expression, this technique is sometimes referred as a "knockdown" to distinguish it from "knockout", in which expression of a target gene is entirely eliminated. Therefore, it is important to determine whether the target gene is being efficiently knocked-down. This type of analysis can be performed by using many different approaches, including immunohistochemistry and western blot experiments, if an antibody against the target protein is available or by RT-PCR analysis, northern blotting or *in-situ* hybridization (Hannon, 2003). Since the phenotypes induced by loss of *cpa* and *cpb* in *Drosophila* were already characterized (Delalle et al., 2005; Janody and Treisman, 2006), I decided to determine whether the UAS-*cpa*-IR and UAS-*cpb*-IR constructs I generated were capable of reproducing the mutant allele phenotypes. The advantage of using this approach is that it allowed me to classify transgenic lines based on the strength of their effects. Since P-element mediated transformation is random, transgenes can be inserted at any place in the genome. Expression of each independent insertion is thus subjected to chromatin state (Lee and Carthew, 2003).

RNAi silencing effect is affected by several factors, which can be manipulated to achieve higher silencing efficiency. Firstly, since the RNAi silencing mechanism is induced through the UAS-Gal4 system, which is temperature sensitive, the manipulation of the temperature allows the analysis of differing levels of depletion. Therefore, the strength of the transgenic lines was analysed at low (22ºC) versus high (27,5ºC) temperature conditions (Tab. 1). In addition, the genetic background of the transgenic flies may also be crucial for the efficient depletion of the RNA. For instance, if the transgenic flies are heterozygous for the gene that is targeted to degradation, only half of the dosage of the RNA will be transcribed. Thus, the RNAi machinery of the transgenic flies operates within half of the cellular dosage of the RNA, which may lead to an increase of its efficiency. This was tested with the UAS-*CP*-IR lines by driving their expression to a Gal4 line recombined with the cpa^{107E} and cpb^{M143} mutations (the results are described below, in section 1.2.3). Moreover, the simultaneous expression of molecular components of the RNAi interference pathway, such as the Dicer enzyme, and the UAS-*CP*-IR transgenic lines is postulated to increase the RNAi silencing efficiency (Mikuma et al., 2004). Therefore, the strength of one of the UAS-*cpa*-IR; UAS-*dicer* transgenic line was determined and the results are briefly presented in Tab. 1 and further described below, in section 1.2.3.

1.2.1 Ubiquitous depletion of *CP*

Animals homozygous mutant for either *cpa or cpb* die at first instar larvae (Janody and Treisman 2006). Therefore efficient depletion of *CP* by using a Gal4 driver expressed ubiquitously, such as *dautherless*-Gal4 (*da*-Gal4) should result in early larval lethality as well. Indeed, when I crossed flies carrying the *da*-Gal4 driver to either independent transgenic flies carrying the UAS-*cpa*-IR and UAS-*cpb*-IR, no progeny could be recovered, suggesting that all transgenic lines can efficiently target *cpa* and *cpb* to degradation (Tab. 1). Only one transgenic line (UAS-*cpa*-IR *C*) gave rise to adult progeny (Tab. 1), suggesting that the transgene is inserted in a region of difficult access to the *da*-Gal4 driver and that its expression level was not sufficient to efficiently deplete *cpa*.

1.2.2 Depletion of *CP* in the eye imaginal disc

In addition, when *cpa* mutant alleles were identified in a mosaic genetic screen for eye patterning genes (Janody et al., 2004) and loss of either sub-units of the *CP* heterodimer induces neuronal degeneration of photoreceptors cells (Delalle et al., 2005). Therefore, driving expression of UAS-*cpa*-IR and UAS-*cpb*-IR specifically in the eye field by using the *eyeless*-Gal4 driver should induce a phenotype, which can be observed in the adult eye. When I drove expression of each UAS-*cpa*-IR transgenic line, by using the *ey*-Gal4 driver, adult eyes appeared to be normal in all independent transgenic lines (Tab. 1). Instead, the UAS-*cpb*-IR transgenic lines were not uniform in the strength of the eye phenotype. While UAS-*cpb*-IR A and B lines appeared to have normal eyes, the UAS-*cpb*-IR C and D lines had a rough eye phenotype (Fig. 2), suggesting loss of *cpb* was severe in the eye. The rough eye phenotype mentioned above was observed in flies raised at 27,5ºC, which is postulated to confer higher levels of depletion but the results of partial depletion of *cpb*, by raising flies at 22ºC were not determined.

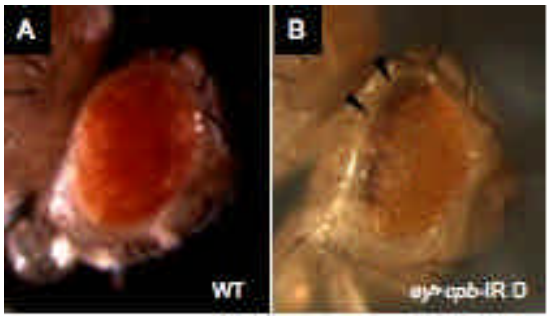


Fig. 2 Directed expression of *cpb*-IR to the eye leads to rough eye phenotype. A) wild type fly B) ey>*cpb*-IR D flies show strong effects of *cpb* depletion when raised at 27,5ºC.

1.2.3 Depletion of *CP* in the wing proper

The phenotype of epithelial extrusion and cell death caused by loss of *CP* in discrete paches in the wing imaginal disc is specific of the wing blade region, which will give rise to the adult wing and is specified by the transcription factor Vg. Moreover, the *CP* inactivation phenotype was shown to be dependant on the presence of Vg (Janody and Treisman, 2006). Thus, depletion of *CP* in *vg* expressing cells should result in an obvious adult wing phenotype, thus providing a measure of the strength of the RNAi efficiency of the UAS-*CP*-IR transgenic lines.

Indeed, wings of two UAS-*cpb*-IR lines (raised at 27,5ºC), namely C and D, were strongly affected by *cpb* depletion (Tab. 1). Not only the wings were reduced and malformed, but also contained thickened veins, suggesting that cell death might have occurred during wing development (Fig. 3). The strength of the effects caused by depletion of *cpb* is similar in both UAS-*cpb*-IR C and D lines. Interestingly, wings of these lines that were raised at 22ºC were significantly less affected by *cpb* depletion: although the wings seemed slightly smaller and were downwardly curved, there was no evidence of apoptosis (Fig 4 B). This shows how temperature affects the level of depletion of *CP*.

To enhance the efficiency of the RNAi mechanism, the *vg*-Gal4 driver was recombined with cpa^{107E} and cpb^{M143} mutations. However, within the lines that presented a wing phenotype (UAS-*cpb*-IR C and D), no detectable differences were observed between the *vg*-Gal4 line and the *vg*-Gal4 line recombined with cpa^{107E} and cpb^{M143}, suggesting that the genetic background of transgenic flies does not play a crucial role in the RNAi silencing effect (since the differences were not significant, the different lines used are not contemplated in Tab. 1).

In contrast to the UAS-*cpb*-IR C and D lines, both the UAS-*cpb*-IR A and B lines and all the UAS-*cpa*-IR lines had normal wings (Tab. 1). This is consistent with the strength of the effects caused by depletion of CP in the *eyless* expression domain, since the lines that present adult wing and eye phenotypes are the same in both situations (UAS-*cpb*-IR C and D), but contrasts to the results obtained when depletion was targeted ubiquitously. This suggests that, even though *cpa* and *cpb* ubiquitous depletion in UAS-*cpb*-IR A, B and UAS-*cpb*-IR A, B is sufficient to lead to lethality, higher levels of depletion are required to obtain eye and wing phenotypes.

Due to the total absence of eye and wing phenotypes in all of the UAS-*cpa*-IR lines, I attempted to increase the RNAi efficiency by establishing a line containing both a UAS-*cpa*-IR and a UAS-dicer (Since the UAS-*dicer* transgene is inserted in the X chromosome, I only used the UAS-*cpa*-IR A whose transgene is in the 3rd chromosome). However, even though ubiquituous targeting of this line led to lethality, no effects were observed by targeting depletion to the *vg* expression domain. This highlights how the

point of the transgene insertion may affect the strength of the RNAi machinery efficiency.

The analysis of the UAS-*CP*-IR phenotypes, through directed expression to *da*-, *ey*- and *vg*-Gal4, was crucial to determine which lines and conditions are preferred to further analyse the effects of *CP* depletion. Therefore, the lines chosen for this purpose were UAS-*cpb*-IR C and D (which have similar strength effects) and the temperature in which transgenic flies were raised was 27,5ºC. Even though the use of the *vg*-Gal4 cpa^{107E} cpb^{M143} line did not seem to affect the RNAi induced silencing effect, this line was chosen to perform experiments whenever *CP* depletion was to be targeted to *vg* expressing cells.

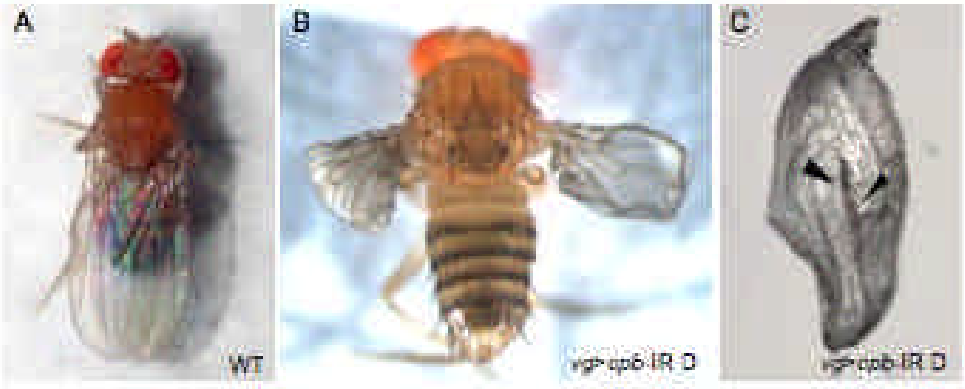


Fig. 3. Directed expression of the *cpb*-IR to the wing leads to reduced and malformed wings. A) wild type fly B) *vg*>*cpb*-IR D fly raised at 27,5 ºC. The wings are reduced and malformed. C) Detail of the wing (of *vg*>*cpb*-IR D transgenic flies raised at 27,5ºC). Wings display thickened veins (indicated by arrows), which suggest cell death may have occurred during its formation.

Tab. 1. Comparison of the relative strength of the effects caused by RNAi treatment, through the use of several drivers. The comparison of the strength of the effects was done according to the temperature conditions to which flies were exposed (22 or 27,5ºC) and is considered to three different drivers, *ey*-,*da*- and *vg*-Gal4. Only two transgenic lines, UAS-*cpb*-IR C and D exhibited strong phenotypes with all the tested drivers. The chromosome location of each transgenic line insertion is also shown.

	Independent insertions		Temperature (ºC)	*da*-Gal4	*ey*-Gal4	*vg*-Gal4
UAS-*cpa*-IR lines	**A**		22	lethal	Not determined	Normal wings
			27,5	lethal	Normal eyes	Normal wings
		UAS-*dicer*	22	Not determined	Not determined	Normal wings
	Ch.: 3th		27,5	lethal	Not determined	Normal wings
	B		22	lethal	Not determined	Normal wings
	Ch.: X		27,5	lethal	Normal eyes	Normal wings
	C		22	viable	Not determined	Normal wings
	Ch.: X		27,5	viable	Normal eyes	Normal wings
UAS-*cpb*-IR lines	**A**		22	Lethal	Not determined	Normal wings
	Ch.: 2nd		27,5	Lethal	Normal eyes	Normal wings
	B		22	Lethal	Not determined	Normal wings
	Ch.: 3th		27,5	Lethal	Normal eyes	Normal wings
	C		22	Lethal	Not determined	Downwardly curved wings (see Fig. 4B)
	Ch.: 2nd		27,5	Lethal	Rough eye	Reduced, malformed wings (see Fig. 3)
	D		22	Lethal	Not determined	Downwardly curved wings (see Fig. 4B)
	Ch.: 2nd		27,5	Lethal	Rough eye	Reduced, malformed wings (see Fig. 3)

1.3 Specificity of the UAS-*CP-RI*

A problem that arises by using the RNAi technique to generate loss of function phenotypes is to prove causality between RNAi treatment and a phenotype. Confirming the transcript levels are reduced is not sufficient to relate the phenotype with the depletion of the target gene, since the RNAi treatment may be silencing one or more genes than the intended target (off-target genes). In order to determine the possible off-targets of the sequence used as IR, I made use of a software available online (http://flyrnai.org/) by the *Drosophila* RNAi screening centre (DRSC). This software allows blasting the combination of an established number of nucleotides (nt) of the submitted sequence with all the annotated genes in the *Drosophila* genome. In *Drosophila*, the small interfering RNA (siRNA), which are responsible for triggering gene silencing, consist normally of duplexes of 21 to 23 nt (Elbashir et al., 2001). Therefore, I blasted every 21-23 nt contained within the UAS-*cpa*-IR and UAS-*cpb*- IR sequences. No predicted off-targets were found by using this technique, suggesting that the phenotypes observed by using the UAS-*cpa*-IR and UAS-*cpb*-IR constructs were due to depletion of *cpa* and *cpb* respectively.

Even though this data accounts for the sequence-specificity of the IR used, it is still *in silico* data. Therefore, it is crucial to demonstrate *in vivo* that the observed phenotype is due to the depletion of *CP*. To do so, I attempted to rescue the *cpb-RI* phenotypes by using flies carrying the *cpb* cDNA under control of the UAS sequences (UAS-*cpb*), which can fully rescue the *cpb* mutant phenotype (Janody and Treisman, 2006). Flies carrying both the UAS-*cpb*-IR and UAS-*cpb* were crossed with flies bearing the *vg*-Gal4 driver. However, the wing phenotypes were indistinguable when compared to control wings of flies *vg>cpb*-IR (data not shown). This suggests that overexpression of *cpb* is not sufficient to rescue the wing phenotype induced by RNAi depletion. Since the UAS-*cpb*-IR targets a sequence of 543 bp of the *cpb* cDNA, it is likely that the *cpb* overexpression construct is also targeted to degradation by the UAS-*cpb*-IR, preventing any suppression effect.

2. Depletion of *cpb* in the wing imaginal disc leads to different outcomes

2.1 Partial depletion of *cpb* in the wing resembles the phenotype of *arc* mutant flies

As referred above, RNAi silencing efficiency is highly dependant on temperature conditions. *vg>cpb*-IR flies raised at 22ºC exhibited downwardly curved wings that seemed smaller than wild type but had no sign of patterning defects (Fig. 4 B). This phenotype resembles the one detected in the wings of flies bearing an *arc* mutation (Fig. 4 C) (Liu and Lengyel, 2000). *arc* encodes an adherens junction-associated PDZ domain protein, expressed in tissues undergoing developmental morphogenetic movements. In third instar wing imaginal disc, *arc* RNA is expressed mainly in the hinge and in the adjacent proximal blade regions (Fig. 4 H) (Liu and Lengyel, 2000). *vg*-Gal4 drives expression of target genes not only in the distal wing blade, where *arc* is not detectable, but also in the hinge domain (Fig. 4 F). This suggests that the downward curved wing phenotype of *vg>cpb*-RI flies raised at 22ºC, is the consequence of *cpb* depletion in the hinge domain. To test this possibility, I targeted depletion of *cpb* only in the hinge region, by driving the expression of UAS-*cpb*-IR to the *30A*-Gal4 driver (Fig. 4 G). However, *30A>cpb*-IR flies had straight wings undistinguishable from wild type (Fig. 4 D). The *30A*-Gal4 driver might induce expression of the UAS-*cpb*-IR at a level too low to result in an obvious phenotype. Alternatively, since *vg*-Gal4 drives expression of target genes in a gradient along the distal proximal axis in the wing blade, weak *cpb* depletion in the proximal part of the wing blade might be sufficient to induce the formation of a downward curved wing.

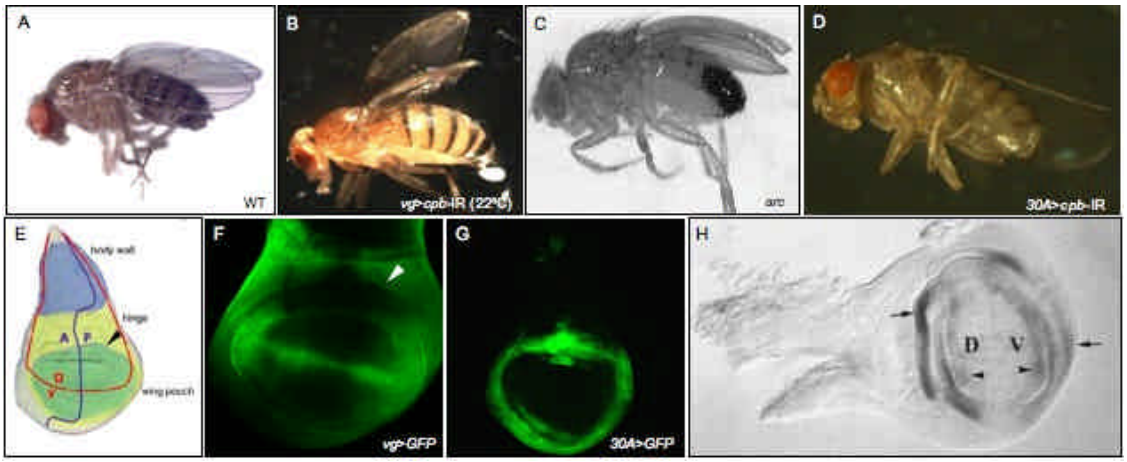


Fig. 4. Partial *cpb* depletion in the wing resembles the wing phenotype of *arc* mutant flies. A) Wild type fly. B) *vg*>*cpb*-IR fly raised at 22ºC. C) *arc* homozygous mutant fly (Adapted from Liu and Lengyel, 2000). D) 30A>*cpb*-IR raised at 27,5ºC (to enhance the phenotype). F) Fate map of the *Drosophila* wing disc. Arrow indicates the proximal region of the wing disc. (Adapted from Butler et al., 2003). F) Expression domain of the *vg*-Gal4 driver (driven with a UAS-*GFP*). G) Expression domain of the *30A*-Gal4 driver (driven with a UAS-*GFP*) H) Expression domain of the gene *arc* (adapted from Liu and Lengyel, 2000). Arrows indicate expression in the prospective hinge and immediately adjacent proximal wing blade regions.

2.2 The local environment influences the behavior of *CP* depleted cells

2.2.1 Depletion of *cpb* in the *vg* expression domain leads to massive cell extrusion and apoptosis

vg>*cpb*-RI flies raised at 22ºC appeared to have smaller wings. This suggests that *cpb*-depleted cells fail to growth. Alternatively, a subset of wing blade cells may have died by apoptosis. Indeed, when the *vg*>*cpb*-IR larvae were raised at 27,5 ºC, adult wings were strongly affected and presented thickened veins that were likely necrosed tissues (Fig. 3 C). In order to determine whether *cpb*-depleted cells in the *vg*-expression domain were undergoing apoptotic cell death, I established a stable line carrying the UAS-*cpb*-IR construct and a UAS-*GFP* reporter gene, allowing me to mark with GFP the *cpb*-depleted cells. Third instar wing imaginal discs from *vg*>*cpb*-RI; *GFP* flies were marked with an anti-Armadillo (Arm) antibody to outline the apical membrane and with an anti-activated Caspase 3 (Casp 3) antibody, witch reveals Caspase-dependent cell death. Indeed, standard confocal sections showed that most of the GFP positive cells in the wing blade epithelium expressed activated Casp 3 (Fig. 5 C), indicating that *cpb*-depleted cells were

dying. In contrast, only few dying cells were recovered in the *vg*-expressing domain of the hinge and notum (Fig. 5 B, C). Cross sections through the wing disc epithelium showed that GFP-positive cells were extruded from the wing blade epithelium and had lost epithelium cell polarity, since they failed to express Arm (Fig. 5 C). However, *cpb*-depleted cells are still maintained in the hinge and notum epithelia (Fig. 5 B, C). Thus, the behavior of *cpb*-depleted cells in the *vg*-expression domain resembles the observed in clones of *cpa* mutant cells (Fig. 5 D), suggesting that clones of *CP* mutant cells die cell-autonomously. However, although depletion of *cpb* in the *vg*-expressing domain is wider (Fig. 5 A) than *cpb* mutant cells induced in discrete patches, a large number of proximal wing blade cells are not targeted by the *vg*-Gal4 driver, and might promote non-autonomous extrusion and death of *cpb*-depleted cells by a process of cell competition.

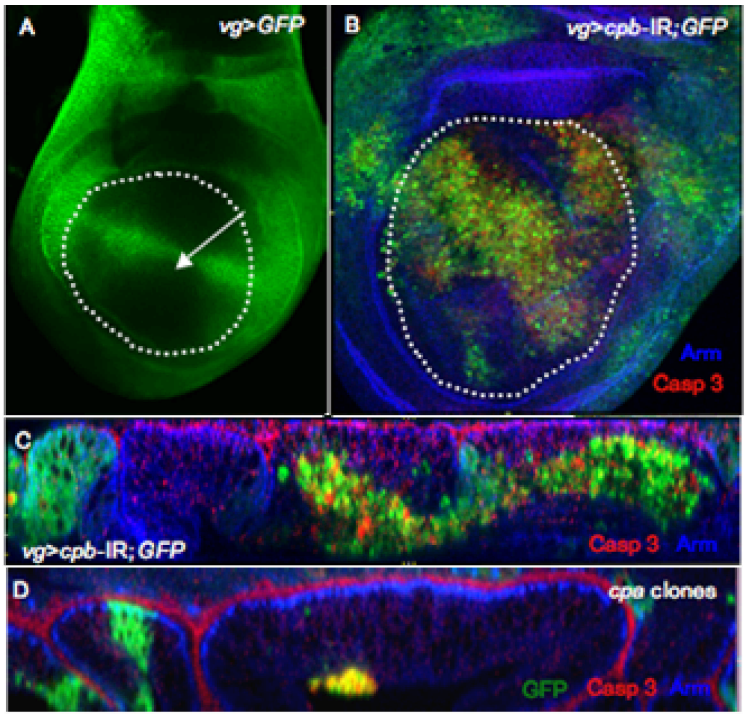


Figure 3. Depletion of *cpb* in *vg*-expressing cells leads to extrusion and cell death. All panels show third instar wing imaginal discs marked with GFP in green and with anti-Arm (blue in B, C and D) and anti-activated Caspase 3 (Red in B, C and D) (A-B) Standard optical sections. (C-E) Optical cross sections. (A-C) *vg*-Gal4 driver driving UAS-*GFP* (A) or UAS-*cpb-RI*, UAS-*GFP* (B and C). D) Clones of *cpa* mutant cells are marked positively by GFP (Image provided by Janody). In A) is presented the *vg* expression domain. *vg* expressing cells targeting depletion of *cpb* undergo massive apoptosis (B,C) and are extruded from the epithelium (C). However, cpb depleted cells are still maintained within the the epithelium in the notum and hinge regions of the disc (C), resembling clones of cells mutant for *cpa* (D).

2.2.2 Broad depletion of *cpb* in the *scalloped* expression (*sd)* domain leads to most of the hallmarks of tumor cells.

With the goal of knocking down *cpb* in the whole wing blade epithelium, I made use of *scalloped*-Gal4 (*sd*-Gal4), which drives expression of a UAS-*GFP* reporter gene in the whole wing blade epithelium, but also in the hinge and part of the notum (Fig. 6 D). *sd>cpb*-IR flies raised at 27,5ºC died at 2nd instar larval stage. By decreasing the temperature to 22ºC, lethality was delayed to pupal stage, allowing me to analyze third instar larval wing discs. Surprisingly, depleting *cpb* in the entire wing blade leads to a completely different outcome from the one that results from *cpb* depletion in the *vg* expression domain: although some *cpb*-depleted cells marked positively by GFP still expressed activated Caspase 3, most of the cells within the wing blade were able to survive (Fig 6 A, C). Not only *cpb* depleted cells are able to survive, but also seem to overproliferate, as suggested by the numerous foldings and overgrown masses of tissue the wing blade contains (Fig. 6 A).

Moreover, in *cpb* depleted cells, the expression of the AJ component Arm is mislocalized (Fig. 6 B), suggesting that epithelial apicobasal polarization is disrupted in these cells. CP have previously been reported to be required for the maintenance of epithelial architecture in clones of *CP* mutant cells (Janody and Treisman, 2006). Loss of apicobasal polarization is considered to be a crucial step in the process of epithelial to mesenchymal transitions (Brumby and Richardson, 2005). Thus, I wondered whether *cpb* depleted cells could show additional features characteristic of EMT. Indeed, closer examination of *cpb* depleted cells in the wing blade, exhibited a morphological difference when compared to wild type cells: *cpb* depleted cells acquired a rounder morphology and seemed to be less adhesive between each other (Fig. 6 D-G). Both disruption of epithelial polarity and altered cell shape characterize EMT, thus the presented data suggest that *cpb* depletion in the wing blade region may induce EMT.

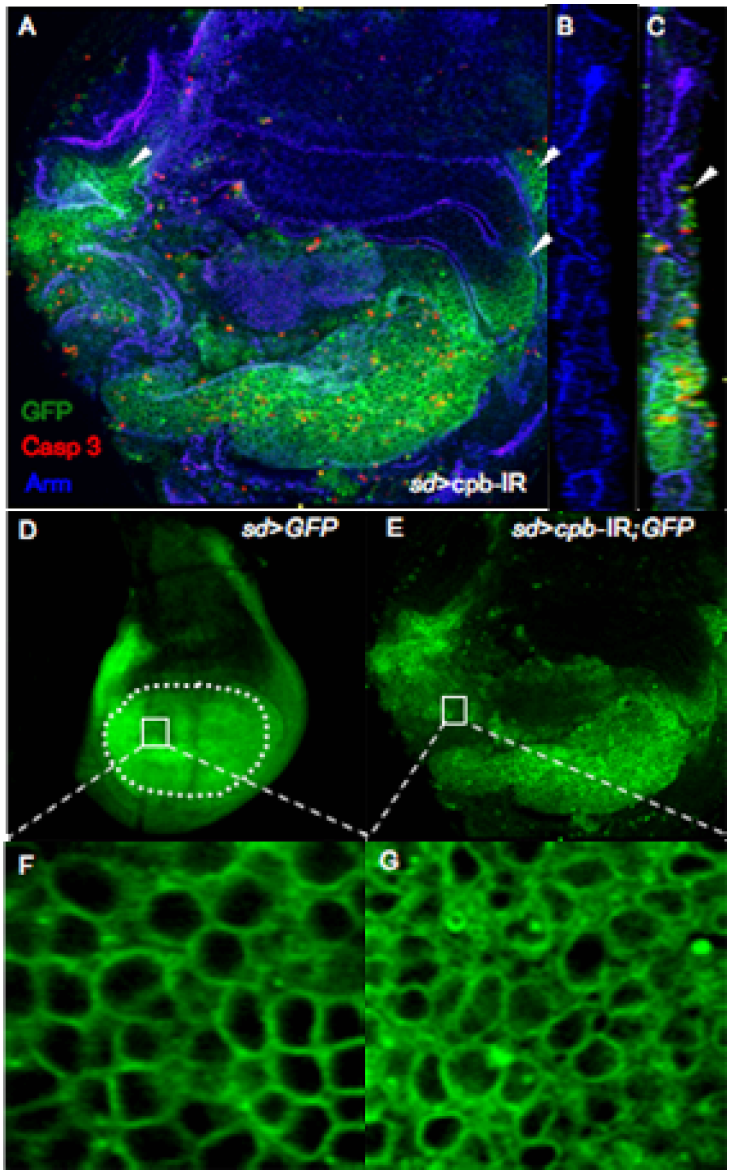


Fig. 6. Broad depletion of *cpb*, targeted to *sd*-expressing cells, leads to most of the hallmarks of tumor development. All panels show third instar wing imaginal discs marked with GFP in green and with anti-Arm (blue in A, B and C) and anti-activated Caspase 3 (Red in A, B and C). (A, D-E) Standard optical sections. (B, C) Optical cross sections. In *sd>cpb*-IR discs only few Casp 3 positive cells are recovered (A, C). Moreover, the disc contains masses of overgrown tissue (A, E) and Arm appears to be mislocalized (B). A closer examination on optical sections of the disc revelead that *cpb* depleted cells are rounder than WT cells (F,G). Even though the phenotype described above is restricted to the wing blade region, since GFP labeled cells are maintained within the epithelia in the remainder regions of the disc (indicated by arrows in A), some displaced cells are often recovered in the hinge region (Indicated by a arrow in C)

EMT transition, resistance to cell death, loss of proliferation control and acquisition of migratory abilites are the four major hallmarks of tumor development. Although some *cpb* depleted cells seem to be recovered in distant regions from the wing blade (indicated by arrows in Fig. 6 C), it is not clear whether they have acquired migratory abilities. Still, most of the hallmarks of tumor cells are present in *cpb* depleted cells.

It is crucial to mention that the phenotype observed in *sd>cpb*-IR discs only occurs in the wing blade region, since in the remainder regions of the disc epithelial cells appear to maintain their characteristics (indicated by arrows in Fig. 6 A).

2.2.3 Wing blade cells depleted of *cpb* seem to acquire the ability to migrate

Previous data on the behavior of *CP* mutant cells in the ovarium follicle epithelium (see Fig. 9 of the Introduction) (Janody, un-published data), have suggested that loss of *cpa* may lead to a migratory type of behavior. Also, either loss of *cpb* in clones or by RNAi depletion in *vg*-expressing cells leads to epithelial extrusion. In addition, *cpb* depleted cells targeted to the *sd* expression domain, were often recovered in regions distant from the presumptive wing blade. Since migration is a hallmark of tumor behavior, confirming that *cpb* depleted cells may acquire migratory abilities would be consistent with this view.

To better understand whether CP might prevent cell movement, I used the *patched*-Gal4 (*ptc*-Gal4) driver, which directs expression of a *UAS-GFP* reporter gene to a few rows of cells along the anterior/posterior (A/P) boundary (Fig. 7 A) (Vidal et al., 2006). As previously, cells targeting *cpb* to degradation were marked with GFP to follow their fate. I assumed that if *cpb*-depleted cells acquire the ability to migrate, they would be found far away from the A/P boundary. Indeed, I found that some of the GFP-positive cells where displaced from the *ptc* expression domain (indicated by arrows in Fig. 6 B). In agreement with previous analysis, *cpb*-depleted cells were extruded basally from the wing blade epithelia, and showed a bright GFP staining, likely due to condensed chromatin, suggesting that these cells were dying (Fig. 6 C, D).

Consistent with previous observations, *ptc>cpb*-RI; *GFP* cells were maintained within the wing hinge and notum. This suggests that *cpb* prevents migration of wing

blade cells. However, we cannot completely exclude the possibility that *cpb*-depleted cells shift far away from where they originate due to a passive extrusion process dependant of apoptotic cell death.

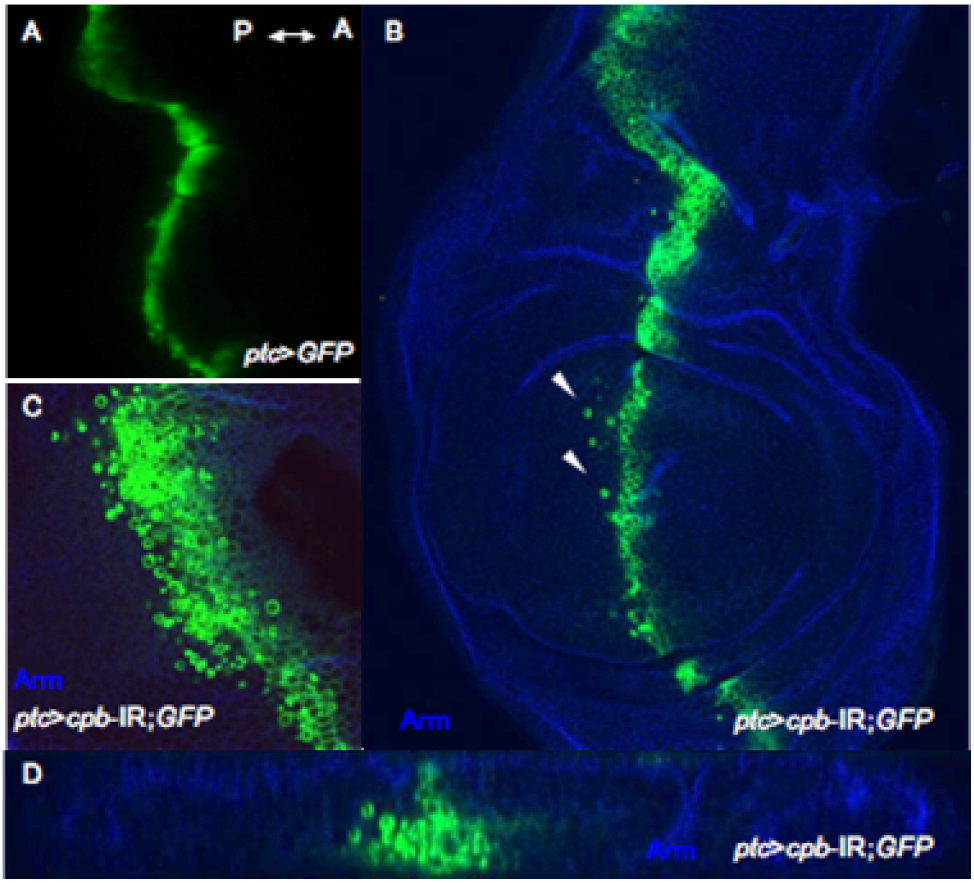


Fig. 4. ***cpb*-depleted cells seem to gain the ability to migrate in the wing blade.** All panels show third instar wing discs, marked by GFP in green and anti-Arm antibody in blue. (A-C) Standard confocal sections. (D) Optical cross section A) *ptc*-Gal4 driving UAS-*GFP* expression (B-D) *ptc*-Gal4 driving UAS-*cpb*-RI, UAS-*GFP*. *cpb* depleted cells, marked posivively by GFP are recovered in distant locations of the A/P boundary, in which *ptc* is expressed (indicated by arrows in B and C). These cells extrude basally (D). C) refers to a basal view of the epithelia, in which a large amount of cells is mislocalized. Note that mislocalized *cpb* depleted cells are mostly recovered in the posterior compartment of the wing disc.

Interestingly, in all *ptc>cpb*-RI; *GFP* discs analyzed, cells were recovered far from the A/P boundary only in the posterior compartment (Fig. 6 B, C, D). *ptc*-Gal4 drives expression in a gradient in which expression is highest in cells apposed to the posterior compartment and falls away gradually in cells within the anterior compartment (Vidal et al., 2006). Since the posterior edge of the *cpb*-depleted cells is directly apposed to wild type cells of the posterior compartment, extrusion, migration and death of *ptc>cpb-RI*, GFP cells might occur through a non-autonomous process due to cell competition. In contrast, in more anterior cells, *cpb*-depletion is graded and does not form a sharp difference between *cpb-depleted* cells and wild type cells. Alternatively, graded depletion of *cpb* in more anterior cells might be insufficient to promote, extrusion, migration and death.

V DISCUSSION

Among the UAS-*cpa*-IR and UAS-*cpb*-IR transgenic lines I obtained, only two transgenic lines of UAS-*cpb*-IR presented a phenotype when crossed with specific Gal4 drivers to measure their strength. (Tab. 1 of the Results). Even though the α subunit of the CP heterodimer caps with higher affinity the barbed end of the actin filament, both α and β subunits are required for the proper function of CP and loss of either one is postulated to result in the same type of phenotype (Wear et al., 2003). Since all the experimental conditions to test efficiency of the RNAi silencing effect were similar for the UAS-*cpa*-IR and UAS-*cpb*-IR transgenic lines and even within the UAS-*cpb*-IR transgenic lines the strength of the effects was highly variable (two lines presented an obvious wing and eye phenotype while the other two didn't), the difference in the strength effects is probably caused by the influence of nearby chromosomal modulation of transgene expression, which depends of the point of transgene insertion (Lee and Carthew, 2003).

Despite the efforts to prove causality between the phenotype of the UAS-*cpb*-IR lines and the RNAi silencing mechanism (by using a UAS-*cpb* construct to rescue the phenotype), the establishment of specificity of the UAS-*cpb*-IR construct is still to be performed. However, several factors account to the fact that the RNAi is targeting specifically *cpb*. First, *in vitro* data using software that finds off-target genes of the *cpb*-IR sequence revealed that, within the *Drosophila* annotated genes, only *cpb* is targeted to degradation. Morevoer, the RNAi induced effects are consistent with the known effects of loss of *cpb*: ubiquitous loss leads to lethality and in restricted regions of the wing disc leads to cell extrusion and death. The most feasible and reliable way to overcome the specificity problem is to perform RNAi using two or more dsRNAs that are directed against the same gene but do not overlap and have little sequence similarity (Hannon, 2003). Recently, the Vienna *Drosophila* RNAi Centre (VDRC) has generated a collection of RNAi transgenic lines that target 85% of the *Drosophila* genome. A UAS-*cpa*-IR and a UAS-*cpb*-IR transgenic lines are available in this collection, and will be used to confirm the phenotype observed with the RNAi constructs I generated.

***CP* mutant cells versus *cpb* depletion by RNA interference: two different outcomes**

In the wing blade epithelia, clones of CP mutant cells (both of *cpa* and *cpb*) suffer basal extrusion and die by apoptosis (Janody and Treisman, 2006). In contrast, in this report I have shown that broad depletion of *cpb* using the RNAi technology results in survival of most cells and overproliferation. Thus, what is leading to these different outcomes?

Two hypotheses may be proposed to explain the contrasting phenotypes of CP inactivation. It can be explained either by a matter of dosage of the levels of *CP* or by a matter of how broad is the epithelia domain in which *CP* is being depleted (Fig. 1).

Firstly, regarding the matter of dosage, the *cpa* and *cpb* mutations used to perform clonal analysis, namely cpa^{107} and cpb^{M143}, are null mutations, meaning that *CP* are not transcribed at all. Consequently, in cells mutant for *CP* there is a complete "knock out" of *CP*. In contrast, the RNAi technology provides a "knock down" of the expression of the target gene, meaning that the depletion is partial, acting like a hypomorph mutation. Even though my results show that the RNAi induced silencing for *cpb* is efficient, it is likely that that reduced levels of *cpb* are still present in the depleted cells.

Differing levels of Src kinase activity are postulated to trigger different cellular responses (Vidal et al., 2007). In this sense, it was shown that while lower levels of activation of Src leads to a weak activation of JNK signaling, directing anti-apoptotic signals and promoting proliferation, overactivation of Src signaling leads to strong JNK activation, inducing apoptotic cell death and invasive migration (Vidal et al., 2007). As I will discuss further, there might exist an association between the activity of the oncogenic family of the Src kinases and CP. We could therefore picture a situation in which the overproliferation phenotype observed in the *sd>cpb*-IR discs could be the result of the partial depletion of *cpb* which, in turn, would lead to low activity of Src. In contrast, in *CP* mutant clones, its total loss would result in a high increase of Src activity, resulting in a strong activation of the JNK pathway and ultimately, in cell death and invasive migration.

Alternatively, the contrasting phenotypes of *CP* inactivation could be a consequence of the extension of *CP* inactivation. In this situation, discrete loss of *CP*, as occurs in clones, would induce wild type cells to activate JNK signaling pathway and

remove abnormal cells from the tissue, through the phenomena of cell competition (Adachi-Yamada and O'Connor, 2004). Since the wild type contribution is removed when the depletion is targeted to a broad domain, as occurs in the case of *sd>cpb*-IR discs, cells depleted of *CP* would be able to survive. This behavior is in agreement with several reports that show that removing the wild type contribution is sufficient for abnormal cells to survive and show an abnormal type of development (Brumby and Richardson, 2003; Vidal et al., 2006).

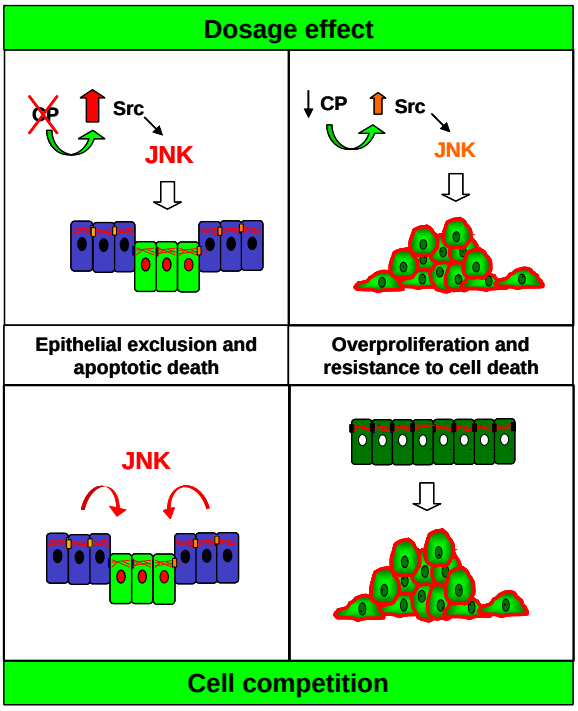


Fig. 1 Schematic representation of the hypotheses proposed to explain the differing behavior caused by *CP* loss of function, by clonal analysis and depletion through RNAi. The cell extrusion *vs*. the overproliferation phenotype can be due to a matter of the Cpb dosage or, in alternative, to cell competition mechanisms. In the first situation, this would lead to different levels of the Src activity, thus activating the JNK signaling pathway. In the second situation, the surrounding wild-type tissue removes abnormal cells through JNK induced apoptosis.

This second hypothesis appears to be the most likely situation to explain the contrasting behavior shown by *CP* mutant clones and RNAi depletion of *CP*. This is reinforced by the fact that RNAi induced *cpb* depletion in restricted wing blade domains, such as in the *ptc* and *vg* expression domains, also induces cell extrusion and cell death. To validate this hypothesis, a simple experiment of clonal analysis using the UAS-*cpb*-IR

transgenic lines to induce clones among wild type tissue (through the use of the MARCM system), could be performed. If, in this situation, clones of UAS-*cpb*-IR are still extruded from the epithelium, I would have evidence to support the cell competition hypothesis.

Cells depleted of *cpb* in the whole wing blade epithelium show most of the hallmarks of tumor cells

Tumor cells display a characteristic set of features that distinguishes them from normal cells. Interestingly, as I have shown, broad depletion of *cpb* in the wing blade region, by inducing its depletion to the *sd*-expressing cells, leads to most of the characteristics of tumor cells:

Epithelial to mesenchymal like transition

In *cpb* depleted cells, the SJ component Arm appears to be mislocalized, indicating epithelial apicobasal polarity may be disrupted in these cells. Disruption of apical basal polarization is crucial to the progression of epithelial cells towards a mesenchymal state (Huber et al., 2005), which implies, among other features, that cells alter cell shape. Indeed, *cpb* depleted cells are morphologically distinct from wild type cells. They are rounder and seem less adhesive to each other. Altogether, these data suggest that *cpb* depleted cells undergo an EMT, however these observations must be further confirmed by staining *cpb* depleted cells with mesenchymal markers, such as N-cadherin and metaloproteases (Weinberg, 2007).

Resistance to cell death

Contrasting to the phenotypes observed when *cpb* depletion was targeted either to the *vg* and *ptc* expressing cells, most of the *cpb* depleted cells in the wing blade were able to survive. This may suggest that these cells acquired resistance to cell death, which is characteristic of tumor cells (Brumby and Richardson, 2005). The fact that some dying cells are recovered does not completely contradict this hypothesis, since there is also a small proportion of dying cells in human tumors (Weinberg, 2007). Alternatively, cell survival can be explained by the fact that cells targeted to depletion in the *sd* expression domain are no longer under the influence of death signaling from surrounding tissue. Resistance to cell death in *Drosophila* is generally mediated by the *Drosophila* inhibitor of apoptosis, Diap 1, which is upregulated in tumor cells (Brumby and Richardson,

2005). Thus, staining *cpb* depleted cells with Diap 1 may show whether they may have acquired resistance to cell death.

Overproliferation

Observations of *cpb* depleted cells in the wing blade suggest that these cells lose their proliferation control and keep dividing continuously. However, to further show increased division in these cells it is necessary to undergo a quantitative type of analysis. Fluorescent Activated Cell Sorting (FACS) analysis may be one way to prove this point, since it provides a method for sorting a heterogeneous mixture of biological cells, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. As *cpb* depleted cells may be marked by GFP, a comparison between the fluorescence of *cpb* depleted cells expressing *GFP* with *GFP* expressing cells in which *cpb* is not targeted to degradation, may show whether loss of *cpb* in a broad domain can lead to loss of proliferation control.

Invasion/metastasis

The role of CP in preventing migration has been suggested before: in the ovarium follicle epithelium clones of *cpa* mutant cells acquire migratory and invasive behavior (Janody, un-published data) and in the wing blade epithelium clones of *CP* mutant cells extrude basally. Also in the present work, I show that depletion of *cpb* in the *vg*, *sd* and *ptc* domains of expression, leads to mislocalization of *cpb* depleted cells. These results are particularly evident when cells are driven to *ptc*-Gal4. However, in this situation it is not clear whether migration is independent of cell death. Indeed, migration seems to occur mostly in the posterior compartment of the disc, in which the levels of *cpb* are directly confronted with the ones of wild type cells. This suggests that neighboring cells activate cell competition mechanisms that ultimately induce cell death of adjacent cells, thus the migration observed would be cell death dependent. A possible way to prove that migration observed in *cpb* depleted cells is caused by loss of *cpb* rather than apoptosis dependent is by blocking cell death using Diap 1, the *Drosophila* inhibitor of apoptosis. A transgenic double line containing both a UAS-*cpb*-IR and a UAS-*Diap1* targeted to the *ptc* domain of expression would be used to corroborate this hypothesis. Another interesting manner of proving invasiveness of *cpb* depleted cells would be to express the activated form of Ras in the *ptc*>*cpb*-IR cells. The combination of the activated form of

Ras with mutations on TSG has been shown to promote invasiveness of cells. In particular, when *scrib* mutant cells are induced in wild type tissue, they are removed by apoptosis, while *scrib* mutant cells (in the same conditions) that simultaneously express Ras^{act} or $Notch^{act}$ acquire an invasive type of behavior (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Again, this experiment could be performed using the UAS-Gal4 system, by expressing simultaneously a UAS-Ras^{act} and a UAS-*CP*-IR line.

An additional feature that characterizes tumor cells is their inability to terminally differentiate. Therefore, it might be interesting to analyze whether these cells show differentiation markers.

Altogether, the data presented strongly suggests that *cpb* prevents tumor development in the wing blade epithelial cells, acting like a tumor suppressor gene. Since Cpb is a component of the CP heterodimer, which requires both α and β subunits to work properly (Wear et al., 2003), depletion of *cpa* should result in the same type of phenotype. Therefore, similar experiments using RNAi mediated analysis can be proposed to demonstrate the tumor suppressor potential of *cpa*.

The postulated tumor suppressor potential of *CP* is restricted to a subset of epithelia

The possible role of CP in preventing tumor development is not a common feature of all type of epithelia, but rather it seems to be specific of only a subset of epithelia. In the ovarium follicle epithelia, *cpa* mutant cells seem to overproliferate and invade distant tissues (Janody, un-published data). As shown in this work, *cpb* depleted cells in the wing imaginal disc epithelia also seem to acquire a tumoral and invasive type of behavior. However, this behavior is restricted to the wing blade, since in the remainder regions of the disc (hinge and notum), *cpb* depleted cells do not seem to be severely affected. This behavior is consistent with the cell death and extrusion phenotype observed for *CP* mutant clones, which is also restricted to the wing blade region. Altogether these data reinforce the idea that in an epithelial tissue continuum, apparently homogenous in cell morphology, not all cells are equivalent in terms of their cytoskeletal architecture and/ or adhesion properties.

The fact that CP has a tissue specific role in preventing tumor formation is very interesting since this is the first time a tumor suppressor gene has been pinpointed to play

such role in a specific subset of epithelia. Moreover, it accounts for the importance of the tissue context during the activation of a tumoral process, which is particularly relevant in humans.

An important question then, is what makes the wing blade region different from the remainder regions of the wing disc. One possible speculation could be a differential junctional composition or strength, between the cells of the different regions of the disc. CP is postulated to localize at the apical membrane, since an HA-tagged form of Cpa co-localizes with most of AJ components (Janody and Treisman, 2006). The Src oncogene also localizes at AJ and its overactivation leads to activation of tumoral processes, through activation of JNK signaling, as already discussed (Yeatman, 2004). Interestingly, the phenotype of *cpb* depletion in the wing blade resembles the phenotypes of *csk* and *dASSP*, both inhibitors of Src activity (Langton et al., 2007; Vidal et al., 2006). Broad depletion of *csk* leads to most of the hallmarks of tumor behavior whereas loss of *csk* as discrete patches leads to epithelial exclusion and apoptotic death (Vidal et al., 2006). dASPP is a positive regulator of Csk, cooperating with it to regulate the activity of Src. *dASPP* homozyguous mutant wing imaginal discs which are simultaneously heterozyguous for *csk*, undergo massive cell death, which seems to occur mostly in the wing blade region (Langton et al., 2007). The phenotypes of both *csk* depletion and *dASPP* loss are a consequence of Src overactivation. Since the referred phenotypes resemble the phenotype of *cpb* depletion, and like Src, *CP* most likely localizes at AJ, the phenotype of *cpb* depletion in the wing blade could be a direct consequence of Src overactivity rather than loss of CP itself. This would lead to Src overactivation, which in turn would dissolve AJ and focal adhesions and activate low levels of the JNK signaling pathway, promoting overproliferation and directing anti apoptotic signals (Fig. 2). The fact that in *cpa* mutant clones the JNK pathway is activated (Janody, un-published data), reinforces this hypothesis. Thus, CP localization at the AJ could be required to prevent Src overactivity specifically in the wing blade, possibly due to slightly different localizations of CP in the different regions.

To further test this hypothesis, double mutant lines for *cpb* and either *src*, *dASPP* or *csk* may be established to determine if this genetic interaction may rescue or enhance the known phenotypes of the single mutants analyzed.

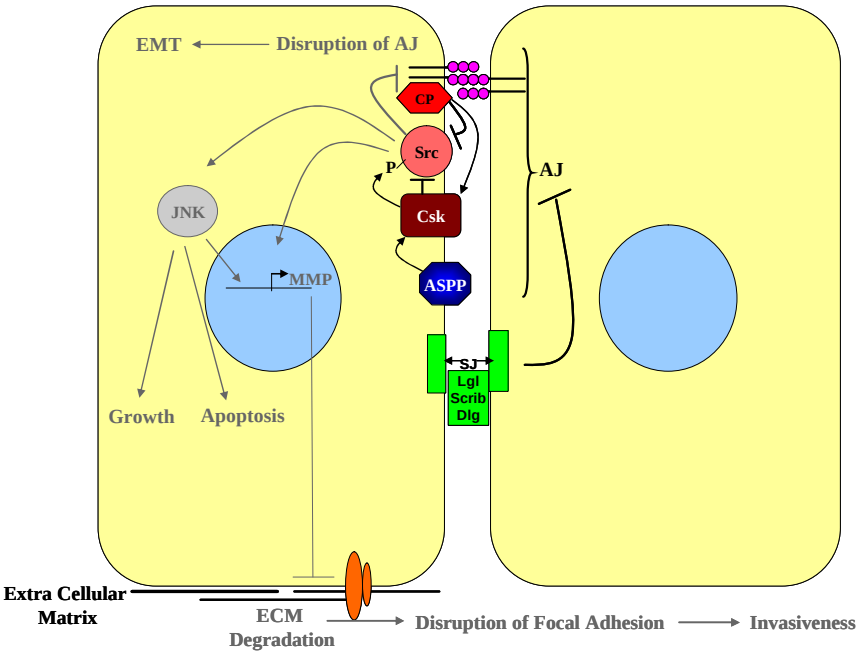


Fig. 2. Loss of *CP* might induce over-activity of Src signaling. The role of CP in tumor development might be due to a possible interaction between this heterodimer and Src. According to this hypothesis, loss of *CP* would lead to the over-activity of Src, which in turn, would activate the JNK signaling pathway, leading either to growth or apoptosis (depending on the levels of activation). Simultaneously, Src would dissolve the AJ and FA, promoting invasiveness.

CP might have additional functions to prevent tumor formation

Both *in vitro* biochemical studies and cultured cells have suggested that the major role of CP is to bind the barbed ends of actin filaments, thereby preventing excessive actin polimerization (Wear and Cooper, 2004). CP function has also been demonstrated in the *Drosophila* epithelia, since loss of CP leads to excessive actin filament accumulation (Janody and Treisman, 2006). Therefore, even though there is no evidence showing that *cpb* depleted cells accumulate actin filaments, it is likely that it occurs. Further experiments, using phalloidin to mark actin filaments in *sd>cpb*-IR discs, must be performed to prove this issue. Considering that the *cpb* depleted cells in the *sd* domain of expression, which behave as tumor cells, accumulate actin filaments, it can be proposed

that tumor formation is induced by the excessive actin polymerization. Thus, the role of CP on tumor prevention would be indirect.

The hypothesis that defects in the actin cytoskeleton may lead to tumor development is consistent with the multiple cellular roles in which actin filaments are involved within the cell. For instance, actin filament turnover is crucial to vesicle trafficking (Apodaca, 2001). Several genes associated with endocytosis were reported to behave as TSG, due to accumulation of important signaling molecules within vesicles (Hariharan and Bilder, 2006). The accumulation of the polarity component Crb within vesicles, leads to neoplastic type of behaviors observed in mutant cells for the endocytic components *avalanche* and *rab5* (Lu and Bilder, 2005). Loss of *CP* could lead to an analogous type of cellular consequence, which would originate a tumor type of behavior. Indeed, preliminary experiments in *cpa* mutant cells suggest that Crb may accumulate within vesicles (Janody, un-published data).

The excessive actin filament polimerization observed in *cpb* depleted cells leads to a dramatic remodeling of the actin turnover in the cell. Actin turnover remodeling has been reported to be crucial during tumorigenesis but is rarely accounted as the principal cause for tumor behavior (Lambrechts et al., 2004). However, we could envision a situation where a dramatic remodeling could itself lead to the acquisition of a tumor type of behavior. There are six actin isoforms in *Drosophila* and in vertebrates, which are highly regulated. In *Drosophila*, it has been reported that the six actin isoforms have restricted patterns of expression in the wing disc (Butler et al. 2003). The tissue specific expression patterns and tight developmental regulation as well as a high conservation across species emphasize the functional importance of the isoforms (Schoenenberg et al., 1999). However, the specific roles of the different actin isoforms have not been determined yet. It would be interesting to further investigate whether CP function may be specific to any actin isoform or whether they are differentially distributed along the imaginal disc tissue or along the apicobasal axis of epithelial cells. In this kind of scenario, loss of CP could induce accumulation of a specific type of actin isoform, thus leading to a specific type of tumoral behavior.

Even though the major known role of CP is to prevent actin filament accumulation, it has been suggested that it may play an additional function in linking actin filaments to

the membrane of cells (Wear and Cooper, 2004). As discussed, a HA-tagged form of Cpa colocalizes with Arm in *Drosophila* epithelial cells, thus CP is postulated to localize at the apical membrane region (Janody and Treisman, 2006). Moreover, both loss of *CP* in clones and by depletion using RNAi leads to mislocalization of Arm, suggesting that CP is required for epithelial maintenance. Disruption of epithelial polarity is a critical step in the progression of cells towards a tumoral behavior. Thus, it can be proposed that CP might play an additional role to that of capping actin filaments, which could be involved either in the establishment and/or maintenance of epithelial polarity, thereby preventing tumor formation.

We could therefore picture a situation in which the role of CP in epithelial establishment/maintenance would be associated with the role of Arc. Arc is an AJ associated protein that contains multiple PDZ domains, thus possibly acts as a scaffold to recruit additional proteins to the cell membrane (Liu and Lengyel, 2000). This hypothesis relies on the fact that both CP and Arc co-localize at AJ, their pattern of expression in the wing imaginal disc overlaps partially (in the proximal regions of the disc) and finally, that the hypomorph phenotype of *arc* and *CP* is similar (downwardly curved wings). Further investigation of the arc loss of function phenotype, for instance through clonal analysis, could provide information on the eventual cooperative interaction of both genes. If the *arc* loss of function phenotype in clones is similar to the one observed in *CP* clones, it can indicate a cooperative interaction between both genes. However, we can not exclude the hypothesis that this interaction might be contrastant rather than cooperative, and therefore analyse the overexpression phenotype of *arc*. In addition, genetic interaction experiments must be performed.

In conclusion, the role of CP in preventing tumour development might be indirect or direct (Fig. 3). Its indirect role would be through its major function, capping of actin filaments. Alternatively, the direct role would be through the hypothesized function of CP in preventing disruption of AJ. Either alternative does not exclude the hypothesis that Src is overactivated due to loss of CP, since it may be a secondary consequence of either of the presented possibilities.

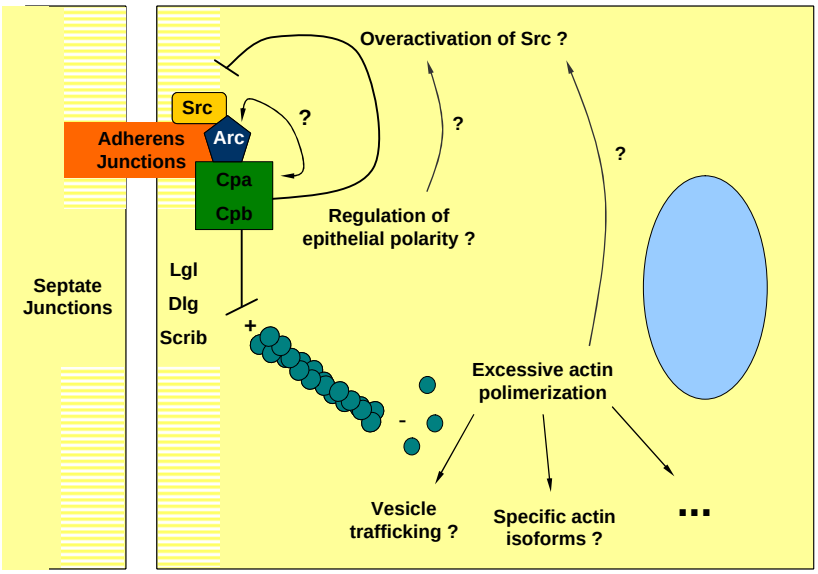


Fig. 3. CP might prevent tumor development through several different mechanisms, which may be direct or indirect. CP may prevent tumor development either through its role in preventing actin filament polymerization, which would lead to a dramatic remodeling of the actin cytoskeleton culminating in a tumoral type of behavior, or through an additional proposed role in the maintenance of the AJ. In this case, it is possible that it might act in a cooperative manner with Arc. Both the hypotheses are consistent with the role of CP in preventing over-activity of Src, since this may be a secondary consequence.

RFERENCES

Adachi-Yamada, T. and O'Connor, M. B. (2004). Mechanisms for removal of developmentally abnormal cells: cell competition and morphogenetic apoptosis. *J Biochem (Tokyo)* **136**, 13-7.

Apodaca, G. (2001). Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton. *Traffic* **2**, 149-59

Baum, B. and Perrimon, N. (2001). Spatial control of the actin cytoskeleton in Drosophila epithelial cells. *Nat Cell Biol* **3**, 883-90.

Bilder, D., Li, M. and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-6.

Brumby, A. M. and Richardson, H. E. (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *Embo J* **22**, 5769-79.

Brumby, A. M. and Richardson, H. E. (2005). Using Drosophila melanogaster to map human cancer pathways. *Nat Rev Cancer* **5**, 626-39.

Butler, M. J., Jacobsen, T. L., Cain, D. M., Jarman, M. G., Hubank, M., Whittle, J. R., Phillips, R. and Simcox, A. (2003). Discovery of genes with highly restricted expression patterns in the Drosophila wing disc using DNA oligonucleotide microarrays. *Development* **130**, 659-70.

Delalle, I., Pfleger, C. M., Buff, E., Lueras, P. and Hariharan, I. K. (2005). Mutations in the Drosophila orthologs of the F-actin capping protein alpha- and beta-subunits cause actin accumulation and subsequent retinal degeneration. *Genetics* **171**, 1757-65.

dos Remedios, C. G., Chhabra, D., Kekic, M., Dedova, I. V., Tsubakihara, M., Berry, D. A. and Nosworthy, N. J. (2003). Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol Rev* **83**, 433-73.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo J* **20**, 6877-88.

Frank, D. J., Hopmann, R., Lenartowska, M. and Miller, K. G. (2006). Capping protein and the Arp2/3 complex regulate nonbundle actin filament assembly to indirectly control actin bundle positioning during Drosophila melanogaster bristle development. *Mol Biol Cell* **17**, 3930-9.

Gibson, M. C. and Perrimon, N. (2003). Apicobasal polarization: epithelial form and function. *Curr Opin Cell Biol* **15**, 747-52.

Hannon, G. (2003) *RNAi: A Guide to Gene Silencing*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Hariharan, I. K. and Bilder, D. (2006). Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet* **40**, 335-61.

Huber, M. A., Kraut, N. and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* **17**, 548-58.

Hug, C., Jay, P. Y., Reddy, I., McNally, J. G., Bridgman, P. C., Elson, E. L. and Cooper, J. A. (1995). Capping protein levels influence actin assembly and cell motility in dictyostelium. *Cell* **81**, 591-600.

Humbert, P., Russell, S. and Richardson, H. (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* **25**, 542-53.

Janody, F., Lee, J. D., Jahren, N., Hazelett, D. J., Benlali, A., Miura, G. I., Draskovic, I. and Treisman, J. E. (2004). A mosaic genetic screen reveals distinct roles for trithorax and polycomb group genes in Drosophila eye development. *Genetics* **166**, 187-200.
Janody, F. and Treisman, J. E. (2006). Actin capping protein alpha maintains vestigial-expressing cells within the Drosophila wing disc epithelium. *Development* **133**, 3349-57.
Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. and Gallant, P. (1999). Drosophila myc regulates cellular growth during development. *Cell* **98**, 779-90.
Johnston, D. (2002) The art and design of genetic screens: *Drosophila* melanogaster. *Nat Rev Genet* **3**, 176-188
Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature* **382**, 133-8.
Klein, T. (2001). Wing disc development in the fly: the early stages. *Curr Opin Genet Dev* **11**, 470-5.
Lambrechts, A., Van Troys, M. and Ampe, C. (2004). The actin cytoskeleton in normal and pathological cell motility. *Int J Biochem Cell Biol* **36**, 1890-909.
Langton, P. F., Colombani, J., Aerne, B. L. and Tapon, N. (2007). Drosophila ASPP regulates C-terminal Src kinase activity. *Dev Cell* **13**, 773-82.
LeBleu, V. S., Macdonald, B. and Kalluri, R. (2007). Structure and function of basement membranes. *Exp Biol Med (Maywood)* **232**, 1121-9.
Lee, Y. S. and Carthew, R. W. (2003). Making a better RNAi vector for Drosophila: use of intron spacers. *Methods* **30**, 322-9.
Liu, X. and Lengyel, J. A. (2000). Drosophila arc encodes a novel adherens junction-associated PDZ domain protein required for wing and eye development. *Dev Biol* **221**, 419-34.
Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat Cell Biol* **7**, 1232-9.
Mikuma, T., Kawasaki, H., Yamamoto, Y. and Taira, K. (2004). Overexpression of Dicer enhances RNAi-mediated gene silencing by short-hairpin RNAs (shRNAs) in human cells. *Nucleic Acids Symp Ser (Oxf)*, 191-2.
Moreno, E., Basler, K. and Morata, G. (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. *Nature* **416**, 755-9.
Pagliarini, R. A. and Xu, T. (2003). A genetic screen in Drosophila for metastatic behavior. *Science* **302**, 1227-31.
Pantaloni, D., Boujemaa, R., Didry, D., Gounon, P. and Carlier, M. F. (2000). The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins. *Nat Cell Biol* **2**, 385-91.
Roberts, D. B. (1998). *Drosophila*, a practical aproach. Oxford University Press
Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23**, 912-23.
Schock, F. and Perrimon, N. (2002). Molecular mechanisms of epithelial morphogenesis. *Annu Rev Cell Dev Biol* **18**, 463-93.
Sundaram, M. V. (2005). The love-hate relationship between Ras and Notch. *Genes Dev* **19**, 1825-39.

Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. (2001). Epithelial cell polarity and cell junctions in Drosophila. *Annu Rev Genet* **35**, 747-84.
Vidal, M., Larson, D. E. and Cagan, R. L. (2006). Csk-deficient boundary cells are eliminated from normal Drosophila epithelia by exclusion, migration, and apoptosis. *Dev Cell* **10**, 33-44.
Vidal, M., Warner, S., Read, R. and Cagan, R. L. (2007). Differing Src signaling levels have distinct outcomes in Drosophila. *Cancer Res* **67**, 10278-85.
Wear, M. A. and Cooper, J. A. (2004). Capping protein: new insights into mechanism and regulation. *Trends Biochem Sci* **29**, 418-28.
Wear, M. A., Yamashita, A., Kim, K., Maeda, Y. and Cooper, J. A. (2003). How capping protein binds the barbed end of the actin filament. *Curr Biol* **13**, 1531-7.
Weinberg, A. (2007) *The biology of cancer*. Garland Pub.

Weng, A. P. and Aster, J. C. (2004). Multiple niches for Notch in cancer: context is everything. *Curr Opin Genet Dev* **14**, 48-54.
Williams, J. A., Bell, J. B. and Carroll, S. B. (1991). Control of Drosophila wing and haltere development by the nuclear vestigial gene product. *Genes Dev* **5**, 2481-95.
Winder, S. J. and Ayscough, K. R. (2005). Actin-binding proteins. *J Cell Sci* **118**, 651-4.
Yamazaki, D., Kurisu, S. and Takenawa, T. (2005). Regulation of cancer cell motility through actin reorganization. *Cancer Sci* **96**, 379-86.
Yeatman, T. J. (2004). A renaissance for SRC. *Nat Rev Cancer* **4**, 470-80.

VII APPENDIX

1. *Drosophila* as a model organism

The fruitfly *Drosophila melanogaster* has been extensively used as a model organism. It was initially chosen because of the short life cycle and easy and cheap maintenance in the laboratory but soon it became one of the favorite models for genetic analysis. The major advantages of *Drosophila* are its reduced genome, the fact that meiotic recombination only occurs in females and the facility to screen for mutations, due to the obvious mutant phenotypes. Furthermore, a large number of developmental processes seem to be conserved between flies and vertebrates (St Johnston, 2002).

1.1 *Drosophila* genetics

The genome of fruit fly has only about 15,000 genes arranged in 4 chromosomes. To perform genetic crosses there are three genetic tools available to use, balancers, phenotypic markers and non-recombination in males (Roberts, 1998).

The balancer chromosomes are chromosomes with one or more inverted segments that suppress recombination and allow lethal mutations to be maintained without selection (lethality as homozygotes). Balancers are also used to maintain chromosomal deficiencies that would otherwise be lethal.

There is large number of phenotypic markers available, both recessive and dominant markers that allow the selection of flies by eye, body, bristle, larval and wing phenotype. By using the Mendel's law and following the dominant phenotypic markers associated with a balancer, it's easy to successfully produce homozygous recessive (non-phenotypic) stock following a multi-generation cross.

The non-recombination in males can be used in certain genetic crosses without worrying about losing the gene of interest by recombination while in the unbalanced state.

1.2 Techniques available in *Drosophila*

1.2.1 FLP/FRT System

To generate mitotic clones usually is used the FLP/FRT system. In a heterozygous parental cell, the site-specific recombinase FLP induces mitotic recombination between FRT sites on homologous chromosome arms. The FLP is expressed under the control of the heat shock promoter. Segregation of recombinant chromosomes at mitosis produces two daughter cells: a mutant cell bearing two copies of the mutant allele and a wild type cell containing only the wild type form of the gene.

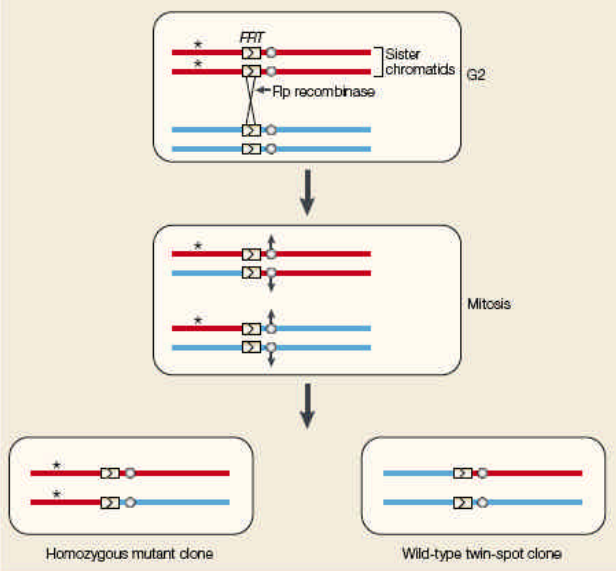


Fig. 1. FRT/FLP system. Adapted from St Johnston, 2002.

1.2.2 The MARCM system

Mosaic analysis with a repressible cell marker (MARCM) is a genetic technique used in *Drosophila* to label single cells or multiple cells sharing a single progenitor. This system is also called GAL80 system and initially contains cells that are heterozygous for a transgene encoding the GAL80 protein, which inhibits the activity of the transcription factor GAL4. Following FLP/FRT mitotic recombination, the GAL80 transgene is removed from the daughter cells, thus allowing expression of a GAL4 driven reporter gene specifically in this daughter cell and its progeny. Only in homozygous mutant cells, in which the GAL80 transgene is not present, will the marker be expressed. By this system the clones are marked positively (Brumby and Richardson, 2005).

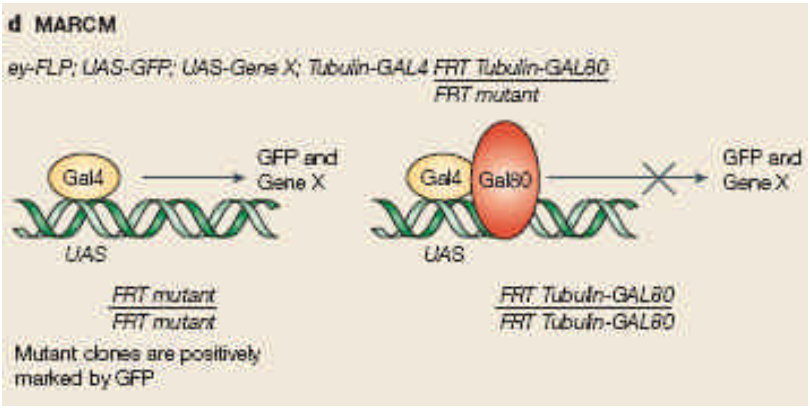


Fig. 1. The MARCM system. Adapted from Brumby and Richardson (2005)

1.2.3 UAS-Gal4 system

The GAL4 system allows the selective expression of any cloned gene in a wide variety of cell and tissue specific patterns in *Drosophila*. A promoter directs expression of the activator GAL4, and the GAL4 in turn directs transcription of the GAL4 responsive UAS target gene. The GAL4 gene and the UAS target are initially separated into two distinct transgenic lines. In the GAL4 line, the activator protein is present, but has no target gene to activate. While in the UAS target gene line, the target gene is silent because the activator is absent. Only when the GAL4 line is crossed with the UAS target gene line that the target gene turned on its progeny.

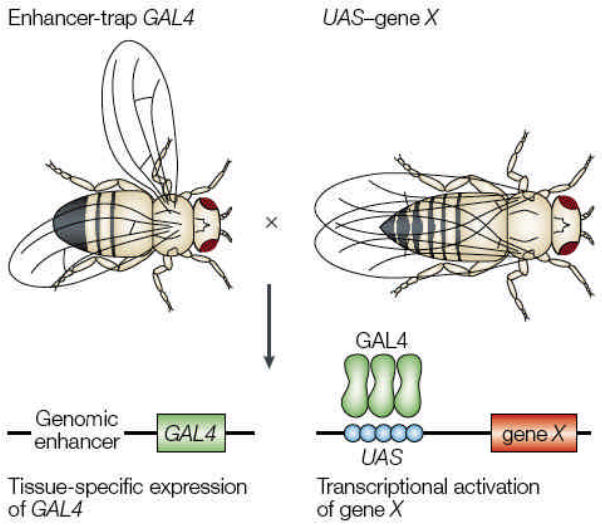


Fig. 3. UAS-Gal4 system. Adapted from (St Johnston, 2002).

1.2.4 P-element mediated transformation

P-element transformation is a method to obtain germline DNA integration following co-injection of pre-blastoderm embryos with purified DNA. The microinjection of the embryos must be performed in the dorsal region of the embryos, in the polar cells, which will give rise to the germ line of the fly. One plasmid encodes the transposase, and the other plasmid contains the inverted repeats flanking the experimental gene. Typically, the marker gene on the P element transformation plasmid encodes an eye color marker (e.g., the rosy gene, ry) that can be used to identify transgenes by virtue of their red eyes (ry+), against a background of non-transformed flies with white eyes (ry-) (Roberts, 1998).

2. *cpa* and *cpb* sequences used as IR

cpa (5'→3')

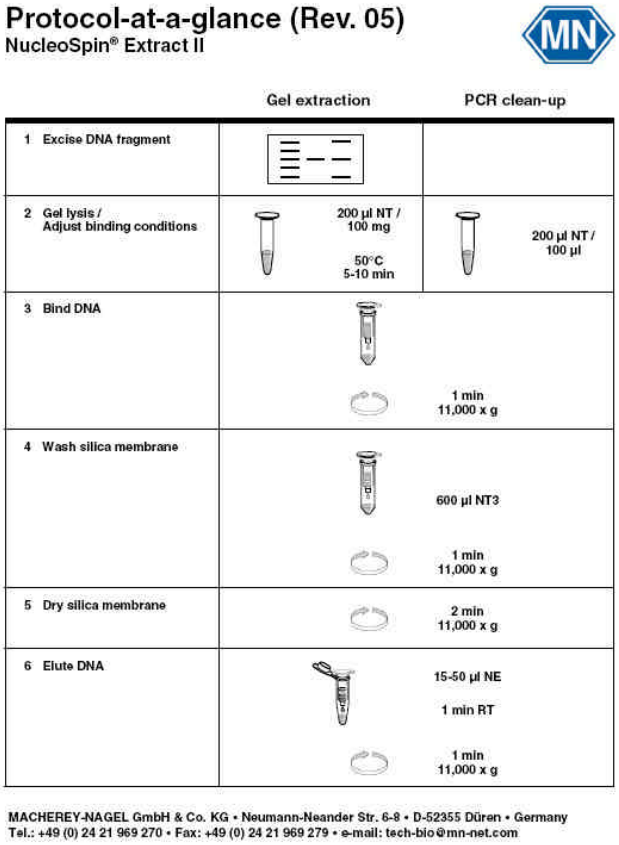
AGCTCTGTTTTTGGAAAGGCGCAGGGCAACCAGATCACCCTGACCATTTGCA
TCGAAGACCACCAGTTCCAGCCGAAGAACTATTGGAATGGCCGATGGCGCTC
CCAGTGGCATGTGACCTTCCAGGCGGGCAGCGGAACCGCCGAACTGAAGGGT
GTGCTCAAGGTGCAGGTGCATTACTACGAGGACGGCAACGTCCAGCTGGTGT
CCTCAAAGGAGTGCCGCGAAAGCGTGGGGTCAGCAACGAGCAGCAGGTGGC
CAAGGAGGTGATCCGTCTGATCGAGGACGCCGAGAATGAGTACCAGCTGGCC
ATTTCGGAGAACTATCAGACGATGTCGGACACGACATTCAAGGCAATGCGTC
GCCAGTTGCCCATCACCAGGACCAAGATCGACTGGAGCAAAATCGTCTCGTA
CAGCATTGGCAAGGAACTGAAGACGCAA

cpb (5'→3')

TCGGAAATGCAGATGGACTGTGCTTTGGATCTGATGCGGAGGCTGCCGCCCC
AGCAGATCGAGAAGAACCTTATTGTCTGATAGACTTGGCACCGGATCTCTGC
GAGGACTTGCTCTCCTCCGTGGACCAGCCGCTGAAGATCGCCAAGGACAAGG
AGCACGGCAAGGACTATCTGCTGTGCGACTATAACCGGGATGGGGACTCCTA
CAGATCGCCCTGGTCGAACTCCTACTATCCGCCGCTGGAGGATGGCCAAATG
CCCTCGGAACGACTGCGCAAACTGGAAATCGAGGCGAACTATGCCTTCGATC
AGTACAGGGAGATGTACTACGAGGGAGGCGTCTCCTCCGTCTCCTATGGGAT
CTGGATCACGGGTTTGCCGCCGTTATACTGATCAAAAAGGCGGGAGATGGCA
GCAAGATGATCCGCGGCTGCTGGGACTCCATCCATGTGGTCGAGGTACAGGA
GAAGACCACCGGCAGGACGGCCCACTACAGCTCACCTCCACGGCAATGCTCT
GGCTGCAGACCAACAAACAGGGT

3. Additional protocols

Protocol of PCR product purification using the NucleoSpin®Extract II (Macherey-Nagel) Kit



Protocol-at-a-glance (Rev. 05)
NucleoSpin® Extract II

	Gel extraction	PCR clean-up
1 Excise DNA fragment		
2 Gel lysis / Adjust binding conditions	200 µl NT / 100 mg 50°C 5-10 min	200 µl NT / 100 µl
3 Bind DNA	1 min 11,000 x g	
4 Wash silica membrane	600 µl NT3 1 min 11,000 x g	
5 Dry silica membrane	2 min 11,000 x g	
6 Elute DNA	15-50 µl NE 1 min RT 1 min 11,000 x g	

Phenol chloroform method

To purify DNA I used the phenol chloroform method (2:1), which implies adding an equal volume of phenol to that of the DNA solution. The aqueous phase that establishes is posterior removed and the procedure is repeated. Finally, an equal volume of cholorophorm is added to the aqueous phase that has been purified with phenol.

Agarose gel

The agarose (Gibco-BRL) was dissolved in TAE 1X, at a final concentration of 1%. 1 µl of etidium bromide (10 mg/ml) was added per 50 ml of TAE (40mM Tris-acetate, EDTA 1mM).